UNIVERSIDADE FEDERAL DO PARANÁ

CAROLINA LOPES LEIVAS

ESTUDO DE POLISSACARÍDEOS DA CARAMBOLA (*Averrhoa carambola* L.) E DA GRAVIOLA (*Annona muricata* L.)

CURITIBA
2015

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Tese apresentada ao Programa de Pós-Graduação em Ciências - Bioquímica, do Departamento de Bioquímica e Biologia Molecular, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências-Bioquímica.

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TERMO DE APROVAÇÃO

CAROLINA LOPES LEIVAS

ESTUDO DE POLISSACARÍDEOS DA CARAMBOLA (*Averrhoa carambola* L.) E DA GRAVIOLA (*Annona muricata* L.)

Tese aprovada como requisito parcial para obtenção do grau de Doutor no curso de Pós-Graduação em Ciências-Bioquímica, Setor de Ciências Biológicas, Universidade Federal do Paraná, pela seguinte banca examinadora:

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“Embora ninguém possa voltar atrás e fazer um novo começo, qualquer um pode começar agora e fazer um novo fim.”

(Chico Xavier)

RESUMO

A carambola (*Averrhoa carambola* L.) é originária do continente asiático e tem sido cultivada em muitos países. Popularmente, os frutos são utilizados no tratamento de várias afecções e estudos científicos relatam atividades antioxidante e hipoglicemiante. A graviola (*Annona muricata* L.) é originária da América Central e do norte da América do Sul. No Brasil é cultivada principalmente nas regiões nordeste, norte e sudeste. Apresenta diversas propriedades medicinais e estudos científicos estão sendo realizados, corroborando com os usos populares. O presente trabalho apresenta a caraterização estrutural de polissacarídeos extraídos dos frutos de *Averrhoa carambola* L. e da polpa dos frutos de *Annona muricata* L. e avaliação da atividade biológica de polissacarídeos purificados. Os polissacarídeos foram extraídos por extrações aquosa e alcalina a quente. Foram fracionados por congelamento/degelo, tratamento com solução de Fehling, cromatografia de troca aniônica, ultrafiltração, e tratamentos com α-amilase, hipoclorito de sódio e dimetilsulfóxido. As moléculas obtidas foram caracterizadas por técnicas químicas, espectrométricas, espectroscópicas (RMN) e cromatográficas (GC-MS e HPSEC). A fração SFSCK, proveniente do extrato alcalino da carambola, apresentou-se composta por uma ramnogalacturonana tipo I com inserções de arabinogalactana tipo I e arabinana. Fucogalactoxiloglucana e heteroxilana foram caracterizadas na fração PFSCK proveniente do extrato alcalino da carambola. A partir do extrato aquoso da carambola foram caracterizadas duas frações contendo arabinogalactana tipo II (frações 50R e 10R) e uma fração (PFSCW) composta por galacturonana substituída. A galacturonana substituída reduziu em 99% a dor em modelo de edema de pata induzido por formalina, na dose de 300 mg/kg, a qual também reduziu o edema de pata em 53%, sugerindo efeito antinociceptivo decorrente do efeito anti-inflamátorio. Os extratos aquoso e alcalino da polpa da graviola apresentaram alta concentração de amido. Foram observadas evidências da presença de arabinogalactana tipo II na fração SGWaH50R e a presença de homogalacturonana linear na fração PGWaH-PD-SR através dos deslocamentos químicos observados nos espectros de RMN-^{13}C. A fração PGKa, proveniente do extrato alcalino da graviola, apresentou-se composta por xilose e os deslocamentos químicos em RMN-^{13}C foram característicos de uma xilana linear β-(1→4)-ligada. A fração PFSGKa, proveniente do extrato alcalino da graviola, apresentou deslocamentos químicos no espectro de RMN-^{13}C similares aos de uma xiloglucana.

Palavras-chave: *Averrhoa carambola* L. *Annona muricata* L. Polissacarídeos. Pectinas. Hemiceluloses. Atividade Antinociceptiva. Atividade Anti-inflamatória.

ABSTRACT

Starfuit (*Averrhoa carambola* L.) is native of Asia and has been cultivated in many countries. In folk medicine, the fruits are used in the treatment of many diseases and scientific studies report antioxidant and hypoglycemic activities. Soursop (*Annona muricata* L.) is native of Central America and northern South America. In Brazil it is grown mainly in the northeast, north and southeast. It has several medicinal properties and scientific studies are being conducted, corroborating the popular uses. This work presents the structural characterization of polysaccharides extracted from the fruits of *Averrhoa carambola* L. and from the pulp of *Annona muricata* L. fruits, and evaluation of the biological activity of purified polysaccharides. The polysaccharides were extracted by hot aqueous and alkaline extractions. Polysaccharides were purified by freeze-thawing, Fehling treatment, anion exchange chromatography, ultrafiltration, and α-amylase, sodium hypochlorite and dimethyl sulfoxide treatments. The obtained molecules were characterized by chemical, spectrometric, spectroscopic (NMR) and chromatographic (GC-MS and HPSEC) techniques. Fraction SFSCK, from the alkaline extract of carambola, was composed of a rhamnogalacturonan I to which a branched arabinan and a type I arabinogalactan are attached. Fucogalactoxyloglucan and heteroxylan were characterized in fraction PFSCK from the alkaline extract of carambola. From the starfruit's aqueous extract two fractions containing type II arabinogalactans (fractions 50R and 10R) and a fraction (PFSCW) containing a substituted galacturonan were characterized. Substituted galacturonan reduced 99% of the pain in a paw edema model induced by intraplantar injection of formalin in the dose of 300 mg/kg, which also reduced the paw edema in 53%, suggesting antinociceptive effect due to anti-inflammatory effect. The aqueous and alkaline extracts of soursop pulp have a high concentration of starch. It has been observed the presence of type II arabinogalactan in fraction SGWaH50R and the presence of a linear homogalacturonan in fraction PGWaH-PD-SR through the chemical shifts present in the ^{13}C-NMR spectra. The fraction PGKa, from the alkaline extract of soursop was composed of xylose and its chemical shifts in ^{13}C-NMR were characteristic of a linear xylan β-(1→4)-linked. Fraction PFSGKa, from the alkaline extract of soursop had chemical shifts in ^{13}C-NMR spectrum similar to a xyloglucan.

Keywords: *Averrhoa carambola* L. *Annona muricata* L. Polysaccharides. Pectins. Hemicelluloses. Antinociceptive activity. Anti-inflammatory activity.

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POLISSACARÍDEOS DA GRAVIOLA

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LISTA DE ABREVEATURAS, SIGLAS E SÍMBOLOS

^{13}C-DEPT	-	*Distortionless Enhancement by Polarization Transfer*
^{13}C-RMN	-	Ressonância magnética nuclear de carbono treze
^{1}H-RMN	-	Ressonância magnética nuclear de próton
AG-I	-	Arabinogalactana tipo I
AG-II	-	Arabinogalactana tipo II
A_pGA	-	Apiogalacturonana
Ara*f*	-	Arabinose furanosídica
Ara*p*	-	Arabinose piranosídica
A_rGA	-	Arabinogalacturonana
$BaCO_3$	-	Carbonato de bário
D_2O	-	Água deuterada
Da	-	Daltons
DA	-	Grau de acetilação
DE	-	Grau de esterificação
DEAE	-	Dietilaminoetil
DMSO	-	Dimetilsulfóxido
DMSO-d_6	-	Dimetilsulfóxido deuterado
Fuc*p*	-	Fucose piranosídica
GaGA	-	Galacturonogalacturonana
Gal*p*	-	Galactose piranosídica
Gal*p*A	-	Ácido galacturônico
GC-MS	-	Cromatografia gasosa acoplada à espectrometria de massa
GGA	-	Galactogalacturonana
Glc*p*	-	Glucose piranosídica
Glc*p*A	-	Ácido glucurônico
GPC	-	Cromatografia de gel permeação
H_2SO_4	-	Ácido sulfúrico
HG	-	Homogalacturonana
HM	-	Pectinas com grau de esterificação superior a 50%
HPSEC	-	Cromatografia de Exclusão Estérica
HSQC	-	*Heteronuclear Single Quantum Coherence*
i.p.	-	Intraperitoneal
IR	-	Índice de refração
kDa	-	Kilodaltons
LM	-	Pectinas com grau de esterificação inferior a 50%

MHz	-	Megahertz
$NaBD_4$	-	Borohidreto de sódio deuterado
$NaBH_4$	-	Borohidreto de sódio
NaCl	-	Cloreto de sódio
NaN_3	-	Azida de sódio
$NaNO_2$	-	Nitrito de sódio
NaOH	-	Hidróxido de sódio
NaOH	-	Hidróxido de sódio
NSAIDs	-	Anti-inflamatórios não esteroidais
RG-I	-	Ramnogalacturonana tipo I
RG-II	-	Ramnogalacturonana tipo II
Rha*p*	-	Ramnose piranosídica
RMN / NMR	-	Ressonância magnética nuclear
TFA	-	Ácido trifluoacético
TOCSY	-	*Total Correlation Spectroscopy*
UA / AU	-	Ácido Urônico
XG	-	Xiloglucana
XGA	-	Xilogalacturonana
Xyl*p*	-	Xilose piranosídica
δ	-	Deslocamento químico

FRAÇÕES DOS FRUTOS DE *Averrhoa carambola*

CW	-	Extrato aquoso
SCW	-	Fração sobrenadante do congelamento/degelo de CW
PCW	-	Fração precipitada do congelamento/degelo de CW
SFSCW	-	Fração sobrenadante de Fehling de SCW
10R	-	Fração retida na ultrafiltração em membrana de 10 kDa de SFSCW
50R	-	Fração retida na ultrafiltração em membrana de 50 kDa de SFSCW
PFSCW	-	Fração precipitada de Fehling de SCW
CK	-	Extrato alcalino
SCK	-	Fração sobrenadante do congelamento/degelo de CK
SFSCK	-	Fração sobrenadante de Fehling de SCK
SFSCK-GAL10	-	Fração precipitada após tratamento com endo-β-(1→4)-D-

galactanase por 10 horas de SFSCK

SFSCK-GAL5 - Fração precipitada após tratamento com endo-β-(1→4)-D-galactanase por 5 horas de SFSCK

SFSCK-GAL5S - Fração sobrenadante após tratamento com endo-β-(1→4)-D-galactanase por 5 horas de SFSCK

PFSCK - Fração precipitada de Fehling de SCK

PFSCK-4M - Fração eluída com 4M NaCl em cromatografia de troca aniônica de PFSCK

XLG - Fração eluída com água em cromatografia de troca aniônica de PFSCK

FRAÇÕES DA POLPA DOS FRUTOS DE *Annona muricata*

GW - Extrato aquoso

GWa - Fração tratada com α-amilase de GW

SGWaH - Fração sobrenadante do congelamento/degelo e do tratamento com hipoclorito de GWa

SGWaH50R - Fração retida na ultrafiltração em membrana de 50 kDa de SGWaH

PGWaH - Fração precipitada do congelamento/degelo e do tratamento com hipoclorito de GWa

PGWaH-PD-SR - Fração sobrenadante do tratamento com resina catiônica e precipitada do tratamento com DMSO de PGWaH

GK - Extrato alcalino

SGK - Fração sobrenadante do congelamento/degelo de GK

SGKa - Fração tratada com α-amilase de SGK

PGK - Fração precipitada do congelamento/degelo de GK

PGKa - Fração tratada com α-amilase de PGK

SFSGKa - Fração sobrenadante de Fehling de SGKa

PFSGKa - Fração precipitada de Fehling de SGKa

SUMÁRIO

1 INTRODUÇÃO

O Brasil é o país de maior biodiversidade do mundo (LIMA, 2007), devido, entre outros fatores, à extensão territorial e aos diversos climas do país. Lorenzi *et al.* (2006) descreveram 827 frutos tropicais para o Brasil e, de acordo com Morton (1987), os frutos dos climas tropicais e subtropicais são conhecidos por estarem associados com muitas propriedades medicinais. Essas propriedades estão relacionadas aos componentes químicos (compostos fenólicos, carotenóides, vitaminas, minerais e carboidratos) presentes nos frutos, os quais estão associados a um risco reduzido de doenças, tais como, doenças degenerativas, doenças cardiovasculares, câncer, entre outras (ALMEIDA *et al.,* 2011). Dentre os frutos escolhidos para o presente projeto, a carambola (*Averrhoa carambola* L.) e a graviola (*Annona muricata* L.) contêm mais de 90% do total de macronutrientes em carboidratos (TACO-UNICAMP, 2011).

A carambola (*Averrhoa carambola* L.) pertence à família *Oxalidaceae* (DASGUPTA; CHAKRABORTY; BALA, 2013; DONADIO *et al*., 2001; MANDA *et al.,* 2012). O fruto é uma baga carnosa, com forma ovóide ou elipsoidal, apresentando cinco gomos longitudinais, sendo consumido preferencialmente *in natura* ou na forma de suco. Popularmente, os frutos da caramboleira são utilizados no tratamento de várias afecções inflamatórias, entre outras (PAYAL *et al.,* 2012). Evidências científicas relatam atividades antioxidante (SHUI e LEONG, 2004) e hipoglicemiante (CHAU; CHEN; LIN, 2004); GUNASEKARA; FERNANDO; SIVAKANESAN, 2011) para os frutos.

A graviola (*Annona muricata* L.) pertence à família *Anonaceae* (PAREEK *et al.,* 2011). O fruto é uma baga, de forma ovóide ou em forma de coração, medindo de 15 a 50 cm de comprimento e 10 a 25 cm de diâmetro, podendo pesar até 6 kg. A graviola apresenta excelentes propriedades medicinais e seus frutos são utilizados tradicionalmente em casos de diarreia (TAYLOR, 2005). Estudos científicos relatam a presença de substâncias

bioativas nos frutos, as quais apresentaram atividade antiproliferativa contra células humanas PC-3 de câncer de próstata (SUN *et al.,* 2014).

Os polissacarídeos de plantas estão sendo amplamente estudados e utilizados em diferentes áreas, tais como, alimentação humana e animal, medicina e farmácia, e na fabricação de papel. O crescente interesse na utilização de polissacarídeos bioativos, deve-se, principalmente à sua biocompatibilidade, biodegradabilidade, baixa toxicidade, e algumas atividades biológicas específicas (LIU; WILLFÖR; XU, 2015). Em nosso Grupo de Pesquisa algumas atividades biológicas já foram testas com diversos polissacarídeos isolados, entre elas, anti-inflamatória (DARTORA *et al*., 2013; RUTHES *et al*., 2013; SCOPARO *et al*., 2013; SILVEIRA *et al*., 2015), anticoagulante (BARDDAL *et al*., 2015; MAAS *et al*., 2012), antinociceptiva, (NASCIMENTO *et al*., 2013; NASCIMENTO *et al*., 2015; RUTHES *et al*., 2013) e gastroprotetora (CANTU-JUGLES *et al*., 2014; CIPRIANI *et al*., 2008; CIPRIANI *et al*., 2009; CORDEIRO *et al*., 2012; MELLINGER-SILVA *et al*., 2011; SIMAS-TOSIN *et al*., 2014). As atividades biológicas dos polissacarídeos estão correlacionadas com as suas características estruturais, sendo necessário a caracterização da estrutura química fina destas moléculas.

Até o momento, não há na literatura estudos que caracterizem a estrutura química dos polissacarídeos da carambola (*Averrhoa carambola* L.) e da graviola (*Annona muricata* L.), nem sobre atividades biológicas que possam estar relacionadas a estes polissacarídeos. Desta maneira, a elucidação da estrutura química fina dos polissacarídeos destes frutos irá contribuir para o aprimoramento do conhecimento científico, através das novas informações sobre propriedades químicas e terapêuticas, além de agregar valor comercial aos frutos e contribuir com o desenvolvimento e a valorização agrícola.

Diante do exposto, este estudo teve como objetivo principal caracterizar estruturalmente os polissacarídeos extraídos dos frutos de *Averrhoa carambola* L. e da polpa dos frutos de *Annona muricata* L. e avaliar a atividade anti-inflamatória e antinociceptiva de polissacarídeos purificados.

2 REVISÃO BIBLIOGRÁFICA

2.1 Averrhoa carambola L.

As carambolas são os frutos da caramboleira (*Averrhoa carambola* L.) e pertencem à divisão Magnoliophyta, classe Magnoliopsida (dicotiledôneas), subclasse Rosidae, ordem Geraniales, família Oxalidaceae (DASGUPTA; CHAKRABORTY; BALA, 2013; DONADIO *et al.,* 2001; MANDA *et al.,* 2012).

É uma frutífera considerada originária do continente asiático (ROZANE, 2008), porém, segundo Manda et al. (2012) o centro de origem da caramboleira foi no Sri Lanka e nas Ilhas Molucas, mas tem sido cultivada no sudeste da Ásia e Malásia por muitos séculos e em áreas tropicais da América, incluindo o Brasil (MANDA *et al.,* 2012; SONCINI *et al.,* 2011). Acredita-se que a caramboleira tenha sido introduzida no Brasil em 1811, através do agrônomo francês Paul Germain, para o extinto Jardim da Aclimação em Olinda, Pernambuco, de onde se difundiu para todo o litoral do país (Granato, 1919[1], citado por ARAÚJO; MINAMI, 2001).

O fruto da caramboleira (FIGURA 1) é uma baga carnosa, com forma ovóide ou elipsoidal, variando no tamanho entre 50-250 mm de comprimento e 30-100 mm de diâmetro, com peso entre 100-250 g. Apresenta cinco gomos longitudinais e, quando cortado, adquire o formato de uma estrela de cinco pontas. A casca, consumida junto com a polpa, é translúcida e sua coloração varia de verde claro ao amarelo intenso, dependendo do grau de maturação. A polpa é translúcida e de consistência firme (SILVA, 1996; DONADIO *et al.,* 2001).

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[1] GRANATO, L. **Cultura da caramboleira**. São Paulo: Pocai & Companhia, 1919. 10p. (Biblioteca Agrícola Granato).

As carambolas são preferencialmente consumidas como fruta fresca ou na forma de saladas, sucos, compotas, doces caseiros e geleias (FERREIRA *et al*., 2008; CRANE, 2013).

Em relação à composição de macronutrientes, a carambola apresenta 91,3% de carboidratos e baixos teores de proteína (7,1%) e lipídeos (1,6%) (TACO-UNICAMP, 2011). Nahar, Rahman e Mosihuzzaman (1990) relataram que a carambola é rica em fibras alimentares, especialmente fibras insolúveis Chau, Chen e Lin (2004) observaram no bagaço da carambola uma predominância de fibra insolúvel (80 g/100 g de fibra dietética total). Dentre os micronutrientes presentes, destacam-se as vitaminas A, C e K (FREITAS *et al.,* 2011), além de ser uma boa fonte de potássio (CRANE, 2013).

FIGURA 1 – FRUTO DA CARAMBOLEIRA (*Averrhoa carambola* L.)
FONTE: O autor (2015)

Na medicina popular, os frutos são recomendados para o tratamento da inflamação da garganta, úlceras na boca, dor de dente, tosse, asma,

soluços, náuseas, vômitos, indigestão, intoxicação alimentar, cólica, diarreia, icterícia, esplenomegalia malárica e ascite (PAYAL *et al.,* 2012). No Brasil, as folhas da caramboleira são utilizadas como diurético, estimulante do apetite, antiemético, antidiarréico e antitérmico. As folhas também são utilizadas topicamente em picadas de insetos (CORRÊA, 1984; MORTON, 1987; IAMONI 1997; MANDA *et al.,* 2012). Além disso, estudos realizados com diferentes partes da planta (frutos, folhas e caules) apresentaram atividades anti-inflamatória (CABRINI *et al*., 2011; SRIPANIDKULCHAI *et al*., 2002), antimicrobiana (SRIPANIDKULCHAI *et al*., 2002), antioxidante (SHUI e LEONG, 2004), antiúlcera (GONÇALVES et al., 2006), hipoglicemiante (CHAU; CHEN; LIN 2004; FERREIRA *et al*., 2008; GUNASEKARA; FERNANDO; SIVAKANESAN, 2011) e hipotensora (SONCINI *et al*., 2011).

2.2 *Annona muricata* L.

A graviola é o fruto da gravioleira (*Annona muricata* L.) e pertence à família Anonaceae, gênero *Annona* (PAREEK *et al*., 2011), tem como centro de origem a América Central e o norte da América do Sul (MORTON, 1987; SILVA, 1996). É encontrada tanto na forma silvestre como cultivada, a partir do nível do mar até altitudes superiores a 1.100 m, distribuídas do Caribe ao sudeste do México e no Brasil. No Brasil, a espécie foi introduzida pelos portugueses no século XVI e distribuída por diversas regiões (MORTON, 1987; FREITAS, 2012) onde tem sido cultivada principalmente nas Regiões Nordeste, Norte e Sudeste (SOBRINHO; BANDEIRA; MESQUITA, 1999). A denominação do fruto da gravioleira varia de acordo com a região, sendo conhecida como *guanábana*, *guanábano, guanavana, guanaba* ou *huanaba* em países de língua espanhola, *soursop* nos países de língua inglesa, *corossol, cachimam épineux, epineux* em países de língua francesa e *sauersack* e *stachelannone* em países de língua alemã (TAYLOR, 2005).

O fruto da gravioleira (FIGURA 2) é uma baga, de forma ovóide ou em forma de coração, medindo de 15 a 50 cm de comprimento e 10 a 25 cm de diâmetro, podendo pesar até 6 kg. A casca é fina de coloração verde-escura e coberta por falsos espinhos carnosos curtos e moles. A polpa é branca, doce, mas ligeiramente ácida, possuindo muitas sementes escuras (SILVA, 1996; LIMA, 2004). Segundo Pareek *et al*. (2011) o melhor sabor do fruto é quando ele está devidamente maduro, apresentando polpa doce e cremosa.

A graviola é um fruto de grande aceitação na agroindústria devido ao seu excelente sabor e ao aroma agradável de sua polpa, podendo ser processada na forma de suco, sorvete, compotas, geleias, doces, xarope, bebida (champola) e néctar. No entanto, grande parte de sua produção é consumida *in natura* (ARAÚJO FILHO *et al*., 1998; SACRAMENTO *et al*., 2003), sendo que o fruto apresenta 85,5% do seu peso em polpa, 8,9% em casca e 3,3% em semente (PAREEK *et al*., 2011).

FIGURA 2 – FRUTO DA GRAVIOLEIRA (*Annona muricata* L.)
FONTE: http://www.brasil.inkasalud.com/index.php/graviola-anticancer

Em relação à composição de macronutrientes, a polpa da graviola apresenta 94% de carboidratos e baixos teores de proteína (4,8%) e lipídeos (1,2%), além de conter 1,9 g/100g de fibra alimentar, apresentando-se como uma boa fonte de energia (62 kcal/100g), vitaminas e minerais (TACO-UNICAMP, 2011). Almeida *et al*. (2011) avaliaram algumas frutas exóticas do Nordeste brasileiro e verificaram que a graviola apresentava um teor moderado de fenólicos totais. Souza *et al*. (2012) analisaram frutos do cerrado brasileiro e relataram atividade antioxidante intermediária e alto nível de carotenóides na graviola. Segundo Almeida *et al*. (2011), alimentos ricos em antioxidantes desempenham um papel fundamental na prevenção de doenças.

A graviola apresenta excelentes propriedades medicinais. Todas as partes da árvore podem ser utilizadas na medicina natural. As folhas podem ser usadas na forma de chás, apresentando propriedades diuréticas, além de serem usadas em uma ampla gama de doenças, incluindo condições inflamatórias, reumatismo, nevralgias, diabetes, hipertensão, insônia, cistite, infecções parasitárias e câncer. Na Amazônia o chá da folha é usado para problemas no fígado. Os frutos e/ou suco são utilizados para febre, diarreia e parasitas e na Amazônia o fruto verde é misturado com o óleo das folhas e azeite de oliva e utilizado topicamente para nevralgia, reumatismo e dores de artrite. As sementes esmagadas são usadas contra parasitas internos e externos e a infusão das sementes moídas é usada para cura da disenteria (TAYLOR, 2005).

Estudo realizado com extrato das folhas indica que a diminuição na concentração de glicose no sangue de ratos diabéticos tratados com *A. muricata* é devido à regeneração e/ou proliferação das células β do pâncreas (ADEYEMI *et al*., 2010). Em outro estudo, Torres *et al.* (2012) avaliaram o efeito de extratos das folhas de graviola sobre o câncer de pâncreas e concluíram que o tratamento com *A. muricata* tem efeitos benéficos sobre o tecido pancreático. Sun *et al*. (2014) isolaram três novas acetogeninas (substâncias bioativas derivadas de ácidos graxos), as quais demonstraram atividade antiproliferativa contra células humanas PC-3 de câncer de próstata.

2.3 POLISSACARÍDEOS DE PLANTAS

A parede celular das plantas apresenta-se como uma estrutura complexa de diferentes macromoléculas ligadas em conjunto, conferindo resistência e forma para a célula, rigidez para a planta, controlando o crescimento celular, participando na comunicação célula-célula e protegendo a célula contra o ataque de patógenos e predadores (BRETT e WALDRON, 1990). Várias camadas formam a parede da célula, são elas: lamela média, parede celular primária e parede celular secundária (REID, 1997). A lamela média forma a interface entre as paredes primárias de células vizinhas. As paredes celulares das plantas são altamente organizadas e compostas por diferentes polímeros, tais como, polissacarídeos, proteínas e substâncias aromáticas, e por água e íons (OCHOA-VILLARREAL *et al*., 2012). A parede celular primária forma-se durante a divisão celular, apresentando importância na expansão celular, e é constituída essencialmente por microfibrilas de celulose envolvidas por uma matriz de polissacarídeos hemicelulósicos e pécticos, e pode conter quantidades significativas de proteínas estruturais e pequenas quantidades de compostos fenólicos, alguns ligados covalentemente aos polissacarídeos (CARPITA e GIBEAUT, 1993; REID, 1997).

De acordo com Carpita e Gilbeault (1993), existem dois tipos distintos de paredes celulares primárias, tipo I e tipo II. A parede celular primária tipo I (FIGURA 3) é observada em dicotiledôneas, classe onde estão incluídos os frutos do presente estudo, sendo composta de aproximadamente 25-40% de celulose, 15-25% de hemiceluloses, 15-40% de substâncias pécticas, 5-10% de proteínas e traços de compostos fenólicos (REID, 1997). Já a parede celular primária tipo II contém quantidades menores de substâncias pécticas e maior quantidade de hemiceluloses (CARPITA e GIBEAUT, 1993; REID, 1997).

A parede celular secundária forma-se após cessar a divisão celular (BURTON; GIDLEY; FINCHER, 2010), sendo bastante espessa devido à deposição de celulose, hemicelulose e lignina, constituindo-se de 40-45% de

celulose, 15-35% de hemicelulose, 15-30% de lignina e traços de substâncias pécticas (DEY; BROWNLEADER; HARBORNE, 1997).

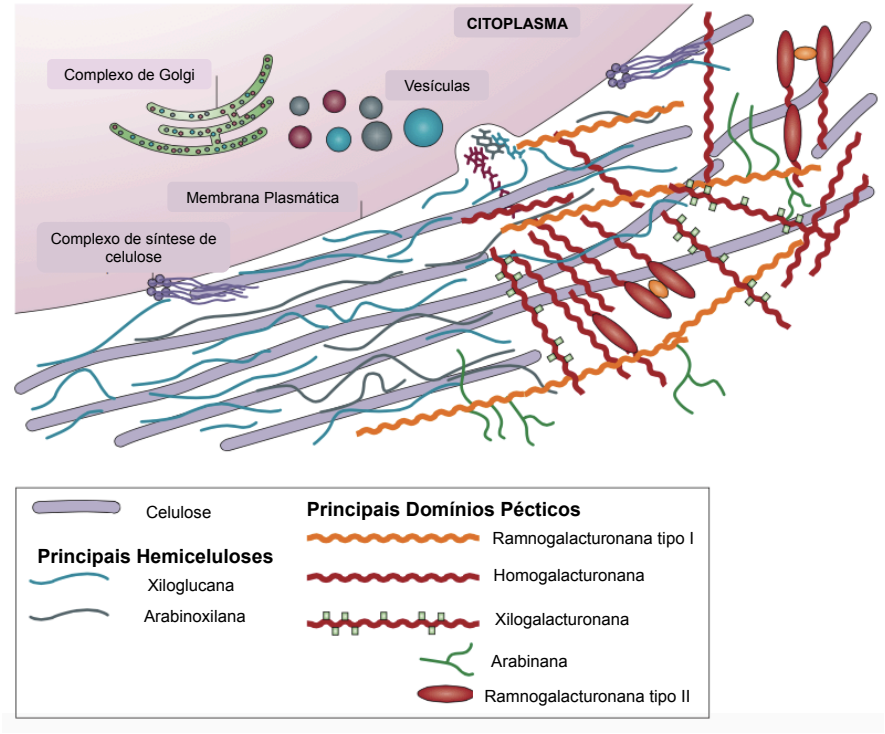

FIGURA 3 – ILUSTRAÇÃO DA PARECE CELULAR PRIMÁRIA DO TIPO I
FONTE: adaptado de COSGROVE (2005)

A seguir serão abordadas algumas características estruturais dos polissacarídeos pécticos e hemicelulósicos presentes em dicotiledôneas.

2.3.1 Polissacarídeos pécticos

Os polissacarídeos pécticos são uma família de polissacarídeos complexos estreitamente associados, presentes em todas as paredes celulares primária e lamela média de células vegetais (VORAGEN *et al.*, 1995), sendo os principais constituintes da parede celular primária tipo I (CAFFAL e MOHNEN,

2009; REID, 1997). Esses polissacarídeos apresentam características comuns, mas são extremamente diversificados em suas estruturas finas (RIDLEY; O'NEILL; MOHNEN, 2001). A característica principal das pectinas são as cadeias lineares de unidades de ácidos galacturônico ligados α-(1→4) (REID, 1997). Segundo Yapo (2011), os polissacarídeos pécticos são: homogalacturonanas (HG), ramnogalacturonanas tipo I (RG-I) e galacturonanas substituídas (ramnogalacturonanas tipo II, xilogalacturonanas, apiogalacturonanas, galactogalacturonanas, arabinogalacturonanas e galacturonogalacturonanas) (FIGURA 4).

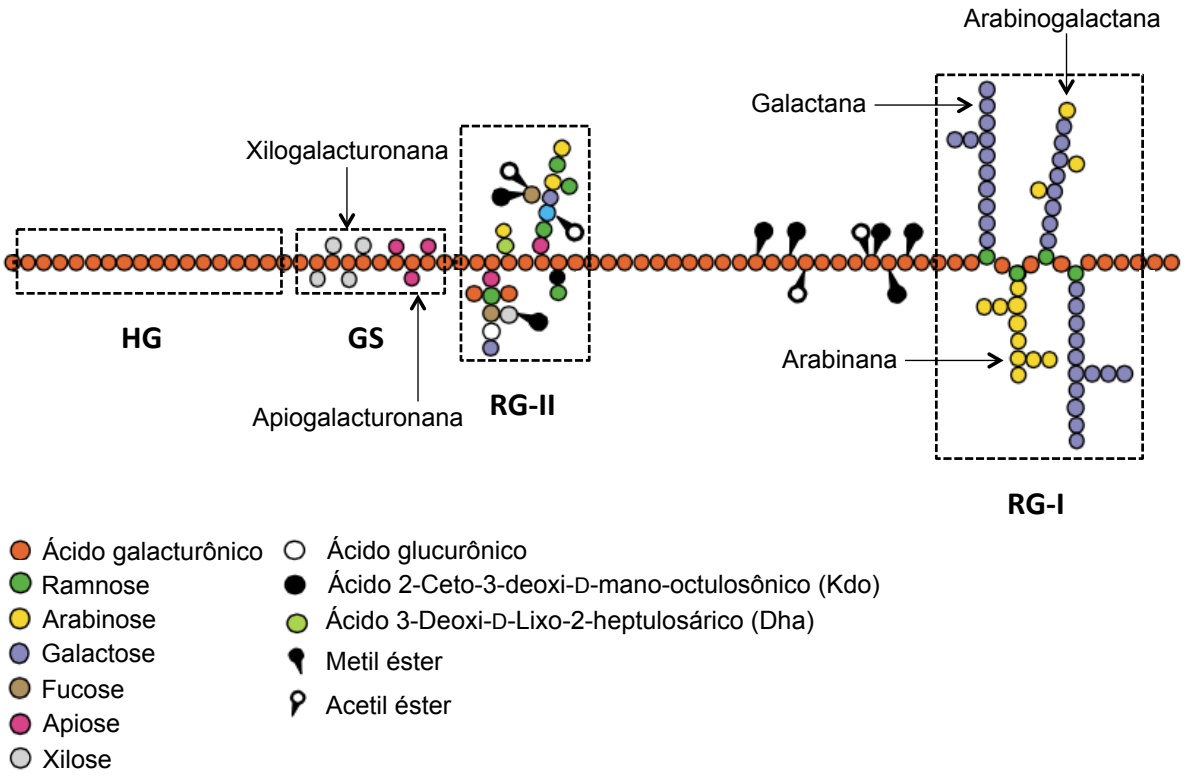

FIGURA 4 – ESTRUTURA ESQUEMÁTICA DOS POLISSACARÍDEOS PÉCTICOS
FONTE: adaptado de WILLATS; KNOX; MIKKELSEN (2006)
NOTA: HG (homogalacturonanas); GS (galacturonanas substituídas); RG-II (ramnogalacturunana tipo II); RG-I (ramnogalacturunana tipo I)

As homogalacturonanas (HG) ou “smooth regions” (FIGURA 5), são formadas por cadeias lineares de unidades de ácidos galacturônicos (Gal*p*A) unidos por ligações glicosídicas do tipo α-(1→4), sendo que algumas dessas unidades de Gal*p*A podem estar parcialmente metil-esterificados em C-6 e acetil-esterificadas em O-2 e/ou O-3 dependendo da espécie (OCHOA-VILLARREAL et al., 2012; RIDLEY; O'NEILL; MOHNEN, 2001). De acordo com Voragen et al. (1995), o grau de esterificação (DE) e o grau de acetilação (DA) são características estruturais importantes nas pectinas. O DE, que indica a porcentagem de grupos carboxílicos metil-esterificados nas pectinas, define as pectinas como pectinas com alto teor de esterificação (HM), as quais apresentam mais de 50% dos grupos carboxílicos metil-esterificados e pectinas com baixo teor de esterificação (LM), que possuem DE inferior a 50%.

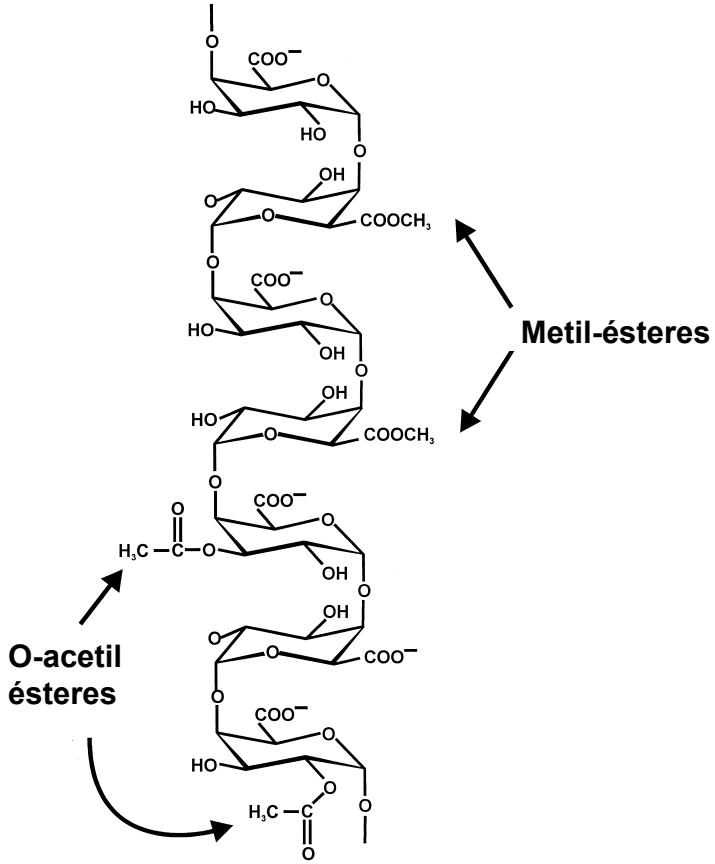

FIGURA 5 – ESTRUTURA DA HOMOGALACTURONANA
FONTE: adaptado de RIDLEY *et al*. (2001)
NOTA: As setas indicam os locais de metil-esterificação e acetil-esterificação

As homogalacturonanas desesterificadas (sem grupos carboxílicos metil-esterificados) são carregadas negativamente e podem interagir ionicamente com Ca^{2+} e, através deste, com outras moléculas de pectinas dando origem às “zonas de junção”, formando um gel estável. Essas zonas de junção podem ser descritas como modelo da “caixa de ovo” (*egg box*) (CAFFAL e MOHNEN, 2009; CARPITA e GIBEAUT, 1993; OCHOA-VILLARREAL *et al*., 2012), representado na Figura 6.

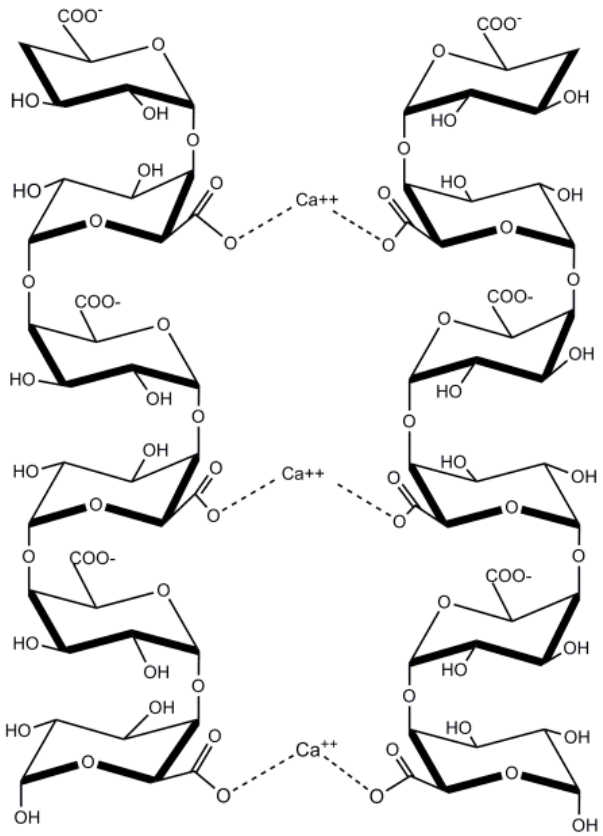

FIGURA 6 – ILUSTRAÇÃO DA INTERAÇÃO DE HOMOGALACTURONANAS COM O CÁLCIO
FONTE: adaptado de OCHOA-VILLARREAL et al. (2012)

As ramnogalacturonanas tipo I (RG-I) ou “hairy regions” (FIGURA 7) são polissacarídeos altamente ramificados com cadeia principal composta por repetições do dissacarídeo [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→] (CARPITA e GIBEAUT, 1993; YAPO, 2011). As RG-I podem ser parcialmente substituídas

em O-4 nas unidades de ramnose por cadeias laterais de açúcares neutros. Existem três tipos principais de cadeias laterais conhecidas, são elas, arabinanas, arabinogalactanas e galactanas (McNEIL *et al*., 1984). No entanto, as cadeias laterais também podem conter resíduos de α-L-Fuc*p*, β-D-Glc*p*A e 4-O-Me-β-D-Glc*p*A (OCHOA-VILLARREAL *et al*., 2012).

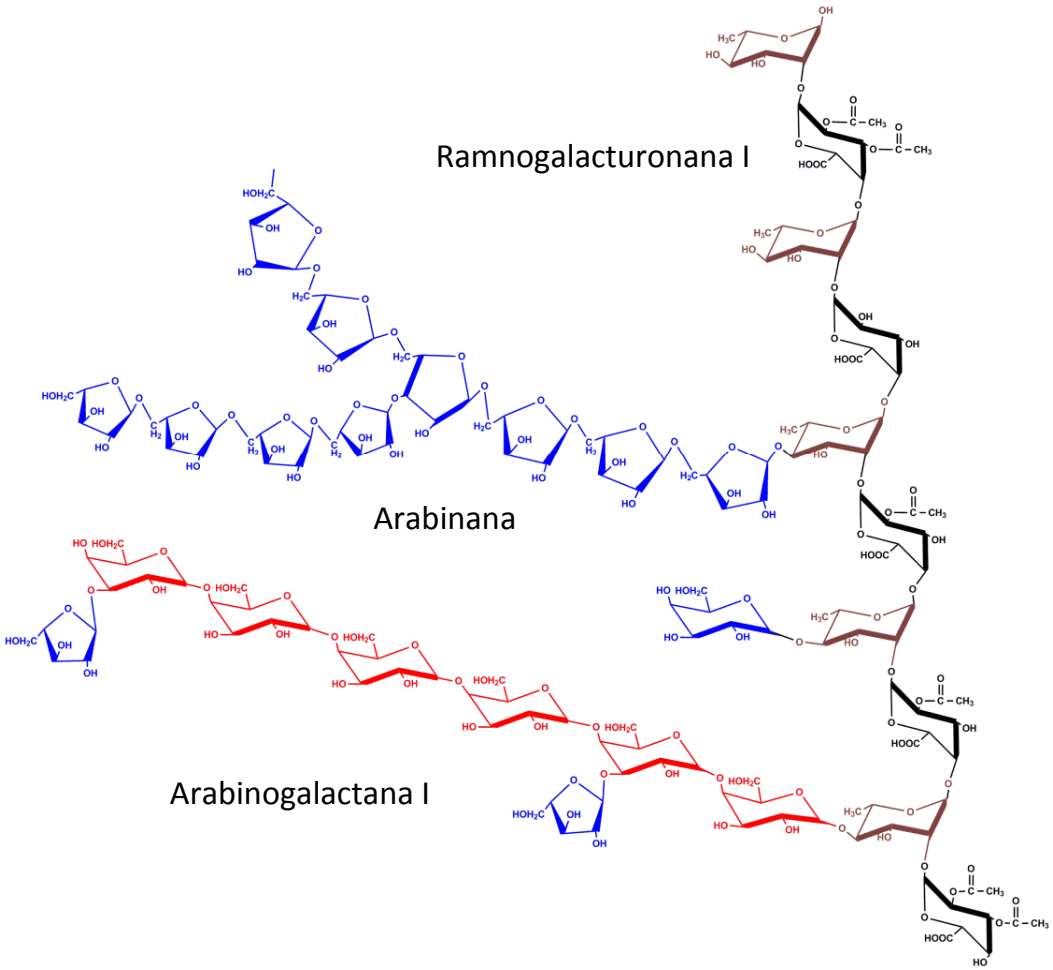

FIGURA 7 – ESTRUTURA DE UMA RAMNOGALACTURONANA TIPO I SUBSTITUÍDA POR CADEIAS LATERAIS DE ARABINANA E ARABINOGALACTANA TIPO I
FONTE: adaptado de OCHOA-VILLARREAL *et al*. (2012)

Arabinanas (FIGURA 7) são polissacarídeos compostos por arabinose, normalmente na forma furanosídica, com cadeia principal de unidades de arabinose unidas por ligações glicosídicas α-(1→5) e substituídas em O-2 e/ou O-3 por cadeias laterais de Ara*f* (REID, 1997; VORAGEN *et al*., 1995).

Arabinogalactanas (AG) ocorrem em dois tipos estruturais diferentes, de acordo com o tipo de ligação das unidades de galactose que compõem a cadeia principal. As arabinogalactanas tipo I (AG-I) (FIGURA 7) apresentam cadeia principal de unidades de galactose β-(1→4) ligada e substituídas em O-3 por unidades ou cadeias laterais de Ara*f.* As AG-I são encontradas em frações pécticas, geralmente ancoradas às RG-I (CARPITA e GIBEAUT, 1993; VORAGEN *et al*., 1995).

As arabinogalactanas tipo II (AG-II) são polissacarídeos altamente ramificados com cadeia principal de unidades de Gal*p* unidas por ligações glicosídicas β-(1→3) e β-(1→6) substituídas em O-3 e O-6 por unidades de Ara*f* (CARPITA e GIBEAUT, 1993; CLARKE; ANDERSON; STONE, 1979; VORAGEN *et al*., 1995). Além de galactose e arabinose, as AG-II podem apresentar diferentes monossacarídeos como ácido glucurônico (GOELLNER *et al*., 2011; WILLFÖR *et al*., 2002; URBAS, BISHOP & ADAMS, 1963), ácido 4-*O*-metil-glucurônico (CIPRIANI *et al*., 2006) e Ara*p* (CAPEK, 2008; GOELLNER *et al*., 2011; ODONMAZIG *et al*., 1994; OLIVEIRA *et al*., 2013; PONDER e RICHARDS, 1997; WILLFÖR *et al*., 2002).

Galacturonanas substituídas (FIGURA 4) são um grupo diverso de polissacarídeos que contém uma cadeia principal de ácido galacturônico ligada α-(1→4) e parcialmente substituída, tais como: ramnogalacturonanas tipo II (RG-II), xilogalacturonanas (XGA), apiogalacturonanas (A_pGA), galactogalacturonanas (GGA), arabinogalacturonanas (A_rGA) e galacturonogalacturonanas (GaGA) (CAFFAL e MOHNEN, 2009; RIDLEY; O'NEILL; MOHNEN, 2001; YAPO, 2011). Ramnogalacturonanas tipo II (RG-II) são polissacarídeos relativamente pequenos, mas com alta complexidade por ser formada por até 12 diferentes tipos de açúcares, incluindo açúcares raros e por mais de 20 diferentes ligações glicosídicas (CAFFAL e MOHNEN, 2009; YAPO, 2011). Xilogalacturonanas são polissacarídeos com cadeia principal de ácido galacturônico ligado α-(1→4) parcialmente substituído em O-3 por unidades terminais de β-D-xilose ou por cadeias laterais de unidades de β-D-Xyl*p* (1→2), (1→3), (1→4), (1→2,3), (1→2,4), (1→3,4) ligadas (YAPO, 2011). Apiogalacturonanas contém resíduos de β-D-Api*f* como cadeias laterais ligadas

à posição O-2 e O-3 das unidades de Gal*p*A da cadeia principal (CAFFAL e MOHNEN, 2009). Galactogalacturonanas apresentam cadeia principal de ácido galacturônico ligado α-(1→4) parcialmente substituído em O-3 por unidades de galactose terminal ou como dissacarídeo (ASPINALL e FANOUS, 1984). Arabinogalacturonanas contém unidades de α-L-Ara*f* como cadeia lateral ligados ao C-3 das unidades de Gal*p*A da cadeia principal (ASPINALL e FANOUS, 1984). Galacturonogalacturonanas apresentam uma cadeia principal de ácido galacturônico ligado α-(1→4) parcialmente substituído em O-2 e O-3 por resíduos de Gal*p*A (OVODOVA *et al*., 2006)

2.3.2 Polissacarídeos hemicelulósicos

Polissacarídeos hemicelulósicos são um grupo heterogêneo de polissacarídeos que apresentam uma cadeia principal formada por açúcares β-ligados e ocorrem em íntima associação com a celulose e tecidos lignificados (OCHOA-VILLARREAL *et al*., 2012), podendo ser extraídos com soluções alcalinas (COSGROVE, 2005). Em dicotiledôneas, grupo onde estão inseridos as espécies estudadas neste trabalho, os polissacarídeos hemicelulósicos compreendem as xiloglucanas, xilanas, mananas e glucomananas, porém, as xiloglucanas são os mais encontrados (OCHOA-VILLARREAL *et al*., 2012; YAPO e KOFFI, 2008).

Xiloglucanas (XG) são formadas por uma cadeia principal composta por unidades de β-D-glc*p* (1→4) ligadas, parcialmente substituída em O-6 por unidades de α-D-xilopiranoses (CARPITA e GIBEAULT, 1993; HAYASHI, 1989; McNEIl *et al*., 1984; RENARD *et al*., 1995). Em dicotiledôneas, as unidades de xilose podem ser substituídas em O-2 por unidades de β-D-Gal*p* e essas unidades de galactose podem ser substituídas em O-2 por unidades de Fuc*p,* sendo o polissacarídedo denominado de fucogalactoxiloglucana, o qual está representado na Figura 8 (HAYASHI, 1989; HSIEH e HARRIS, 2009; McNEIL *et al*., 1984; RENARD *et al*., 1995).

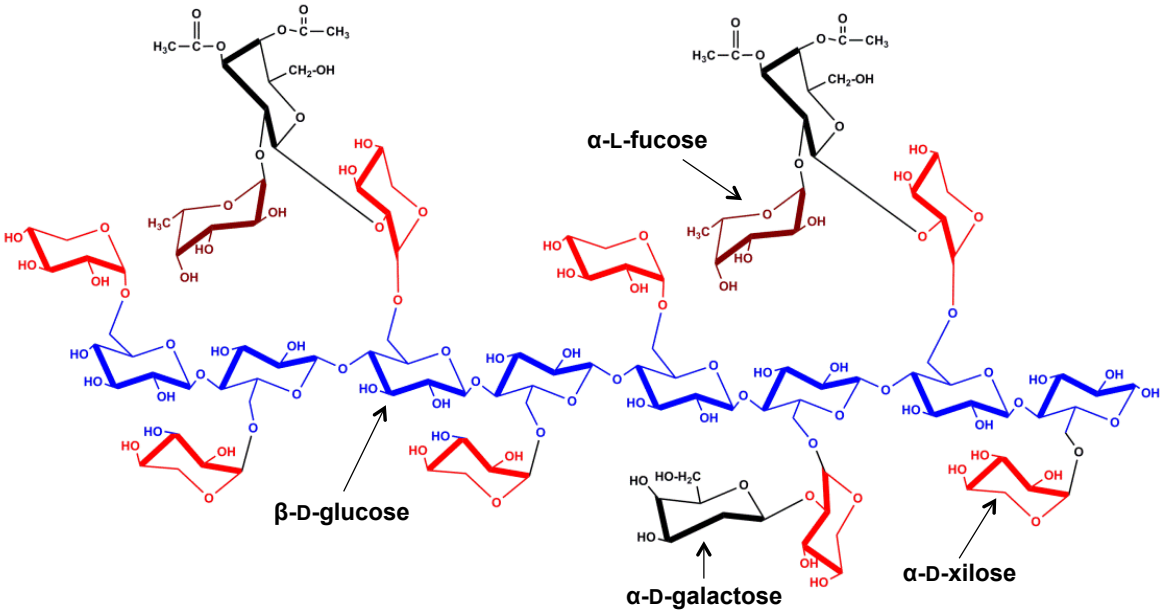

FIGURA 8 – ESTRUTURA DE FUCOGALACTOXILOGLUCANA
FONTE: adaptado de OCHOA-VILLARREAL *et al*. (2012)

Dependendo da fonte, as xiloglucanas podem conter cadeias laterais diferentes. Uma abordagem utilizada para identificar essas cadeias laterais é o uso de enzimas específicas que produziram oligossacarídeos. Esses oligossacarídeos podem ser caracterizados recebendo um código, formado por uma única letra (FIGURA 9) (HSIEH e HARRIS, 2012). Essa nomenclatura foi primeiramente desenvolvida por Fry *et al*. (1993) e continham 8 códigos diferentes e, mais recentemente, foram acrescentados novos códigos, conforme Figura 9.

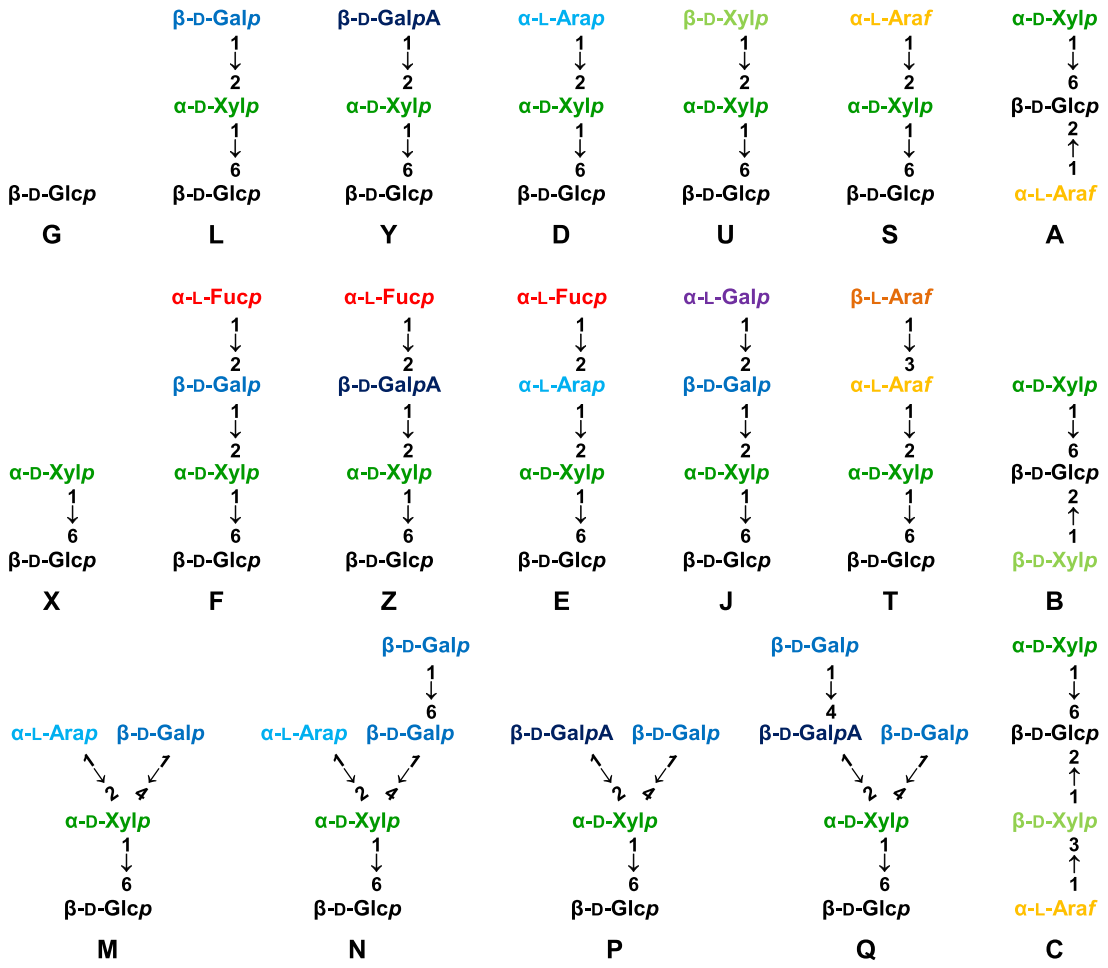

FIGURA 9 – ESTRUTURA QUÍMICA E NOMENCLATURA DE ALGUMAS CADEIAS LATERAIS DE XILOGLUCANA
FONTE: adaptado de TUOMIVAARA *et al*. (2015)

As xilanas (homoxilanas, heteroxilanas neutras e heteroxilanas ácidas) formam um grupo diverso de polissacarídeos formados por uma estrutura comum de unidades de β-D-Xyl*p* (1→4) ligadas. As heteroxilanas apresentam suas cadeias principais substituídas em O-2 e/ou O-3 por diferentes grupos ou cadeias laterais (OCHOA-VILLARREAL *et al*., 2012). Esses grupos podem ser Glc*p*A, 4-*O*-Me-Glc*p*A, Ara*f* e Ara*p* (CIPRIANI *et a*l., 2008; NASCIMENTO *et al*., 2013; SIMAS-TOSIN *et al*., 2014).

2.4 POLISSACARÍDEOS DE FRUTAS

A seguir serão descritos os polissacarídeos identificados em alguns frutos (QUADRO 1). Observa-se que até o momento não há nenhum estudo que caracterize os polissacarídeos das frutas aqui estudadas (carambola e graviola).

ESPÉCIE VEGETAL	**POLISSACARÍDEOS**	**REFERÊNCIAS**
Actinidia deliciosa (kiwi) *Actinidia chinensis* Planch. "Hort16A (kiwi amarelo)	AG-I, AG-II, fucogalactoxiloglucana, HG, RG-I, RG-II, XG e xilana ácida	KATO *et al*., 2001; REDGWELL, MELTON; BRASCH, 1988; SAUVAGEAU *et al*., 2010
Ananas comosus (abacaxi)	AG-I, AG-II, fucogalactoxiloglucana, glucuronoarabinoxilana, HG e RG-I	KATO *et al*., 2001; SMITH e HARRIS, 1995; YAPO, 2009
Argania spinosa (argania)	Amido, RG-I, RG-II e XG	ABOUGHE-ANGONE *et al*., 2008
Armeniaca siberica (damasco)	Arabinana, HG metil- e acetil-esterificadas, RG-I e xilana ácida	ODONMAZIG *et al*., 1990; 1992
Citrus depressa (tangerina de Taiwan)	AG-I, AG-II, HG metil-esterificada e RG-I	TAMAKI *et al*., 2008
Citrus limon (limão)	AG-I, AG-II, HG e RG-I	YAPO, 2009
Citrus sinensis (laranja)	Arabinana, AG-I, HG, heteroxilanas, heteromananas, RG-I, RG-II e XG	PRABASARI *et al*., 2011
Dimocarpus longan Lour. (longan)	Heteroxilana	JIANG *et al*., 2009
Diospyros kaki (caqui)	Arabinana, AG e fucogalactoxiloglucana	ASGAR; YAMAUCHI; KATO, 2004; CUTILLAS-ITURRALDE *et al*., 1998; ITO e KATO, 2002
Eriobotrya japonica L. (nêspera)	AG, amido, RG-I, xilana e XG	FEMENIA *et al*., 1998
Fragaria annassa (morango)	AG, amido, fucogalactoxiloglucana e RG-I	ITO e KATO, 2002; LEGENTIL *et al*., 1995
Malus domestica (Maçã)	AG-I, arabinogalacturonana, fucogalactoxiloglucana, galactoglucomanana, glucuronoarabinoxilana, RG-I, xilogalacturonanas	ASPINALL e FANOUS,1984; KATO *et al*., 2001; OECHSLIN; LUTZ; AMADO, 2003; RAY *et al*., 2014; VRIES *et al*., 1983
Mangifera indica (manga)	AG, amido, HG metil-esterificada, RG-I e XG	IAGHER; REICHER; GANTER, 2002; YASHODA; PRABHA; THARANATHAN, 2005
		continua

Mauritia flexuosa (buriti)	Amido, arabinana linear e ramificada, HG metil-esterificada, homoxilana, RG-I e α-glucana (1→3)-(1→4) ligada	CANTU-JUNGLES *et al*., 2015; CORDEIRO; ALMEIDA; IACOMINI, 2015
Morinda citrifolia (noni)	Arabinana, AG-I, HG, heteroxilanas, heteromananas, RG-I, RG-II e XG	BUI; BACIC; PETTOLINO, 2006
Musa spp. (banana)	AG-I, AG-II, fucogalactoxiloglucana, HG e RG-I	ITO e KATO, 2002 YAPO, 2009
Myrtus cammunis (murta)	AG-I, RG-I e XG	CHIDOUH; AOUADI; HEYRAUD, 2014
Olea europaea (azeitona)	Xilana e XG	VIERHUIS *et al*., 2001
Opuntia ficus-indica (figo-da-índia)	Amido, arabinana ramificada, heteroxilana e RG-I	HABIBI; MAHROUZ; VIGNON, 2002; 2005
Orbignya phalerata (babaçu)	α-glucana (1→4) ligada com ramificações em O-3 e O-6	SILVA e PARENTE, 2001
Passiflora edulis (maracujá)	AG-I, AG-II, HG e RG-I	SILVA *et al*., 2012; YAPO, 2009
Paullinia cupana (guaraná)	Amido, arabinana, heteroxilana, HG metil- e acetil-esterificadas e RG-I	DALONSO e PETKOWICZ, 2012
Persea Americana (abacate)	Fucogalactoxiloglucana	KATO *et al*., 2001
Prunus domestica (ameixa)	AG-I, arabinana linear, fucogalactoxiloglucana, galactana linear, HG metil- e acetil-esterificadas e RG-I	CANTU-JUNGLES *et al*., 2014; ITO e KATO, 2002; POPOV *et al*., 2014
Prunus dulcis (amêndoa)	Arabinana ramificada	DOURADO *et al*., 2006
Prunus persica (pêssego)	AG-II, fucogalactoxiloglucana e HG	KATO *et al*., 2001; SIMAS-TOSIN *et al*., 2012
Psidium guajava L. (goiaba)	AG-I, AG-II, RG-I xilana e XG	MARCELIN; WILLIAMS; BRILLOUET, 1993
Solanum betaceum (tamarillo)	AG-I, amido, arabinana linear, galactoarabinoglucuronoxilana e HG altamente metil-esterificada	NASCIMENTO *et al*., 2013; 2015
Spondias cytherea (cajá)	AG-I, amido e RG-I	IACOMINI *et al*., 2005
Theobrama grandiflorum (cupuaçu)	Amido, HG metil-esterificada e RG-I	VRIESMANN e PETKOWICZ, 2009
Vaccinium myrtillus (mirtilo)	AG-I, AG-II, RG-I e XG	HILZ *et al*., 2005; 2007
Vitis vinífera (uva)	AG-I, AG-II, arabinana, fucogalactoxiloglucana, HG, RG-I e RG-II	DOCO *et al*., 2003; SAULNIER; BRILLOUET; JOSELEAU, 1988; VIDAL *et al*., 2001
Ziziphus jujuba Mill. (jujuba)	Arabinana, galactana, HG metil-esterificada e RG-I	ZHAO *et al*., 2006

QUADRO 1 – POLISSACARÍDEOS IDENTIFICADOS EM FRUTOS
FONTE: O autor (2015)
NOTAS: AG – arabinogalactana; HG – homogalacturonana; RG – ramnogalacturonana; XG – xiloglucana.

2.5 ATIVIDADE BIOLÓGICA DE POLISSACARÍDEOS DE FRUTAS

Os polissacarídeos podem ser obtidos de diferentes fontes naturais, incluindo as frutas, e apresentam uma variedade de atividades biológicas, as quais podem ser influenciadas pelas características estruturais dos polissacarídeos, como composição monossacarídica, grau de substituição, tipos de ligação glicosídica e massa molar (LIU; WILLFÖR; XU, 2015). A seguir serão descritas algumas atividades biológicas testadas com polissacarídeos isolados de frutas (QUADRO 2).

ATIVIDADE BIOLÓGICA	**POLISSACARÍDEOS / ESPÉCIE VEGETAL**	**REFERÊNCIAS**
Anticoagulante e antitrombótico	HG (*Citrus sinensis* L.)	MAAS *et al*., 2012
Anti-inflamatória	α-glucana (*Orbignya phalerata*)	SILVA e PARENTE, 2001
	Galactomanoglucana (*Arecastrum romanzoffianum*)	SILVA e PARENTE, 2010a
	Galactoarabinoglucuronoxilana (*Solanum betaceum*)	NASCIMENTO *et al*., 2013
	HG e RG-I (*Prunus domestica*)	POPOV *et al*., 2014
Antioxidante	Polissacarídeo neutro (*Litchi chinensis* Sonn.)	YANG *et al*., 2006
	Polissacarídeo bruto (*Physalis alkekengi*)	GE *et al*., 2009
	Pectina (*Paullinia cupana*)	DALONSO e PETKOWICZ, 2012
	Polissacarídeo ácido (*Mangifera pajang*)	AL-SHERAJI *et al*., 2012
	Polissacarídeo ácido (*Carica papaya* L.)	ZHANG *et al*., 2012
	Polissacarídeo neutro (*Camellia oleifera* Abel)	JIN, 2012
	HG e RG-I (*Prunus domestica*)	POPOV *et al*., 2014
Antinociceptiva	AG-I (*Solanum betaceum*)	NASCIMENTO *et al*., 2013
Gastroprotetora	Galactomanoglucana (*Syagrus oleracea*)	SILVA e PARENTE, 2010b
	AG-I ancorada na RG-I e uma fração bruta (*Prunus domestica*)	CANTU-JUNGLES *et al*., 2014
Hepatoprotetora	Polissacarídeo ácido (*Hovenia dulcis*)	WANG *et al*., 2012
	Polissacarídeo ácido (*Malus domestica*)	YANG *et al*., 2013
	Polissacarídeo ácido (*Zizyphus jujube*)	LIU *et al*., 2015
Imunoestimulante	Galactomanoglucana (*Syagrus oleracea*)	SILVA e PARENTE, 2010b

QUADRO 2 – ATIVIDADE BIOLÓGICA DE POLISSACARÍDEOS DE FRUTAS
FONTE: O autor (2015)
NOTAS: AG-I – arabinogalactana tipo I; HG – homogalacturonana; RG-I – ramnogalacturonanas tipo I.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Caracterizar estruturalmente os polissacarídeos extraídos dos frutos de *Averrhoa carambola* L. e da polpa dos frutos de *Annona muricata* L. e avaliar a atividade anti-inflamatória e antinociceptiva de alguns polissacarídeos purificados.

3.2 OBJETIVOS ESPECÍFICOS

Visando atingir o objetivo geral descrito, os seguintes objetivos específicos foram delineados:

- Extrair os polissacarídeos dos frutos de *Averrhoa carambola* L. e da polpa dos frutos de *Annona muricata* L. utilizando extração aquosa e alcalina;

- Purificar os polissacarídicas extraídos;

- Caracterizar a estrutura química fina dos polissacarídeos purificados;

- Investigar a atividade anti-inflamatória e antinociceptiva de polissacarídeos purificados e caracterizados.

ARTIGO I

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Structural characterization of a rhamnogalacturonan I - arabinan - type I arabinogalactan macromolecule from starfruit (*Averrhoa carambola* L.)

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ABSTRACT

A structural characterization of polysaccharides obtained from edible tropical fruit named starfruit (*Averrhoa carambola* L.*)* was carried out. After fractionation by freeze-thaw and Fehling precipitation, a pectic polysaccharide was obtained. It was composed of rhamnose, arabinose, galactose and uronic acid in the 5.0: 72.5:12.1:10.4 molar ratios, respectively. A combination of monosaccharide, GPC, methylation and NMR analysis and enzymatic hydrolysis with endo-β-(1→4)-D-galactanase showed the presence of a rhamnogalacturonan I to which a branched arabinan and a type I arabinogalactan are attached. The arabinan moiety was formed by (1→5)-linked α-L-Ara*f* units in the backbone, branched only at *O*-3 by (1→2)- and (1→3)-linked α-L-Ara*f* units, while the type I arabinogalactan was formed by (1→4)- and (1→4,6)-linked β-D-Gal*p* units in the backbone with (1→5)-, (1→3,5)- and (1→3)-linked α-L-Ara*f* units as side chains.

Keywords: Starfruit, Type I Rhamnogalacturonan, Arabinan, Type I Arabinogalactan, *Averrhoa carambola*.

1 Introduction

Pectins are a family of closely associated complex polysaccharides present in all plant primary cell walls and intercellular regions of higher plants (Voragen, Pilnik, Thibault, Axelos & Renard, 1995). They have common features, but are extremely diverse in their fine structures (Ridley, O'Neill & Mohnen, 2001). Homogalacturonan (HG), rhamnogalacturonan I (RG-I) and substituted galacturonans such as rhamnogalacturonan II (RG-II), xylogalacturonan (XGA), apiogalacturonan (A_pGA), galactogalacturonan (GGA), arabinogalacturonan (A_rGA) and galacturonogalacturonan (GaGA) are the pectic polysaccharides in all primary cell walls that have been studied. Rhamnogalacturonan I is composed by a backbone with repeating units of alternating rhamnose (Rha) and galacturonic acid (GalA) linked [→4-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→]. The side chains, mainly linked to the *O*-4 of some of the α-L-Rha*p* units, could be arabinans, galactans, type I and/or type II arabinogalactans (AG) (Yapo, 2011).

RG-I have been reported in some fruits up to now, as for example, in apple cellulosic residue (Oechslin, Lutz & Amadò, 2003), in caja fruits (*Spondias cytherea*) (Iacomini et al., 2005), in citrus peels (Yapo, Lerouge, Thibault & Ralet, 2007), in the pulp of *Argania spinosa* (Aboughe-Angone et al., 2008), in albedo cell walls of orange (*Citrus sinensis*) (Prabasari, Pettolino, Liao & Bacic, 2011) and in prunes (*Prunus domestica*) (Cantu-Jungles et al., 2014).

Averrhoa carambola L., also known as starfruit or carambola, is an edible tropical fruit of the Oxalidaceae family, it has an oblong shape with three to six longitudinal ribs, resulting in a star-shaped cross-section when cut. This specie is native of Malaysia and cultivated in various other Asian countries and the tropical areas of America, including Brazil (O'Hare, 1993; Soncini et al., 2011; Manda, Vyas, Pandya, & Singhal, 2012).

Carbohydrates are the main macronutrient present in starfruit (Manda et al., 2012), however, there is no studies dealing with the identification and chemical characterization of starfruit's polysaccharides. Thus, this paper reports the isolation, composition and structural features of a pectin, formed by a

rhamnogalacturonan I to which a branched arabinan and a type I arabinogalactan are attached.

2 Materials and methods

2.1 Plant material

Ripe fruits of starfruit (*Averrhoa carambola* L.) from cultivar B10 were purchased in a local market in Curitiba, State of Paraná, Brazil.

2.2 General analytical methods

All solutions were evaporated below 60 °C under reduced pressure. Dialysis were performed using 12-14 kDa cut-off membrane.

The total lipid quantification was performed by extraction employing chloroform-methanol (1:1) as solvent through Soxhlet apparatus.

The fraction was carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972), using $NaBH_4$ as the reducing agent, giving products with the –COOH groups of its uronic acid residues reduced to – CH_2OH.

2.3 Extraction and purification of polysaccharides

The fruits were washed, cut and the seeds were manually removed. The fruits were freeze-dried and milled. Dried pulp powder (310.7g) was defatted with chloroform-methanol (1:1), yielding a moisture and nonpolar compounds content of approximately 88% and 4%, respectively. Polysaccharides were extracted from the residue with boiling water under reflux for 2 h (x 6, 1 L each). The aqueous extracts were obtained by centrifugation (12000 x *g*, 20 min at 10ºC), combined and concentrated under reduced pressure. The polysaccharides were precipitated with ethanol (3 vol.), collected

by centrifugation (12000 x *g*, 20 min at 10ºC), and freeze-dried, giving fraction CW (7% yield).

The remaining residue was then extracted with aq. 10% NaOH under reflux at 100ºC for 2 h (x6, 1 L each) in the presence of $NaBH_4$. The alkaline extracts were obtained by centrifugation (12000 x *g*, 20 min at 10ºC), neutralized with acetic acid (HOAc), dialyzed against tap water, concentrated under reduced pressure and freeze-dried, giving fraction CK, with 5% yield (Fig. 1). A freeze-thaw treatment was applied in fraction CK, to give cold-water soluble and insoluble fractions SCK and PCK, respectively. In this procedure, the sample was frozen and then thawed at room temperature followed by centrifugation, until no more precipitate appeared.

Fraction SCK was dissolved in distilled water and then treated with Fehling's solutions (Jones & Stoodley, 1965), resulting in a soluble fraction SFSCK and a precipitated fraction PFSCK, which were separated by centrifugation (12000 x *g*, 20 min at 10 °C). Each fraction was neutralized with acetic acid (HOAc), dialyzed against tap water and deionized with cation exchange resin.

The yields of polysaccharides were expressed as % based on weight of dried starfruit that was submitted to extraction (310.7g).

2.4 Monosaccharide analyses

Neutral monosaccharide components and their ratios were determined after hydrolysis of the polysaccharides with 2 M trifluoroacetic acid (TFA) for 8 h at 100 °C, followed by conversion to alditol acetates by reduction with $NaBH_4$ and acetylation with acetic anhydride-pyridine (1:1, v/v, at 100 °C for 30min). These were analyzed by GC–MS using a Varian gas chromatograph and mass spectrometer, model Saturn 2000R, with He as carrier gas. A capillary column (30 m x 0.25 mm i.d.) of DB-225, held at 50 ºC during injection for 1 min, then programmed at 40 ºC/min to 220 ºC and held at this constant temperature for 19.75 min. was used for the quantitative analysis.

The uronic acid contents were determined using the m-hydroxybiphenyl colorimetric method (Filisetti-Cozzi & Carpita, 1991), using galacturonic acid as standard.

2.5 Determination of homogeneity and molecular weight of polysaccharides

The homogeneity and molecular weight of water soluble polysaccharides were determined by gel permeation chromatography (GPC), using a refractometer and a ultraviolet detector (at 280 nm) as detection equipments. Four columns were used in series, with exclusion sizes of 7 x 10^6 Da (Ultrahydrogel 2000, Waters), 4 x 10^5 Da (Ultrahydrogel 500, Waters), 8 x 10^4 Da (Ultrahydrogel 250, Waters) and 5 x 10^3 Da (Ultrahydrogel 120, Waters). The eluent was 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 ml/min. The samples, previously filtered through a membrane (0.22 μm, Millipore), were injected at a concentration of 1 mg/ml. To obtain the molecular weight, standard dextrans (487kDa, 266kDa, 124kDa, 72.2kDa, 40.2kDa, 17.2kDa and 9.4kDa, from Sigma) were employed to obtain the calibration curve. The molecular weight of the samples were calculated according to the calibration curve.

2.6 Methylation analysis of polysaccharide

Prior to glycosyl linkage analysis, uronic acids were reduced using carboxyl reduction method of Taylor and Conrad (1972). Then the carboxyl-reduced sample was *O*-methylated according to the method of Ciucanu & Kerek (1984), using powdered NaOH in DMSO-MeI. The per-*O*-methylated polysaccharide was then submitted to methanolysis in 3% HCl–MeOH (at 80 °C, 2 h) followed by hydrolysis with H_2SO_4 (0.5M, 12 h, at 100°C) and neutralization with $BaCO_3$. The resulting mixture of partially *O*-methylated monosaccharides was successively reduced with $NaBD_4$ and acetylated with acetic anhydride-pyridine. The products (partially *O*-methylated alditol acetates) were examined by capillary GC-MS. A capillary column (30 m x 0.25 mm i.d.) of DB-225, held at 50 ºC during injection for 1 min, then programmed at 40 ºC/min

to 210 ºC and held at this temperature for 31 min was used for separation. The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005; Sassaki, Iacomini, & Gorin, 2005).

2.7 Nuclear magnetic resonance (NMR) spectroscopy

13C {1H} NMR spectra were acquired at 50 °C on a Bruker AVANCE III 400 NMR spectrometer, operating at 9,5 T, observing 13C at 100.61 MHz, equipped with a 5-mm multinuclear inverse detection probe with z-gradient. The water soluble samples were acquired in D2O. Chemical shifts were expressed as δ ppm relative to CH3 signal from acetone at δ 30.2 as internal reference.

2.8 Enzymatic hydrolysis with endo-β-(1→4)-D-galactanase

Fraction SFSCK was degraded with an endo-β-(1→4)-D-galactanase (EC Nº 3.2.1.89) from *Aspergillus niger* (Megazyme International, Ireland). A sample material (100 mg) was dissolved in 30 ml 0.02M sodium acetate buffer (pH 4.4) and incubated with 75 µl enzyme solution at 45ºC, at two incubation times (5 h and 10 h). The enzyme was inactivated at 100ºC for 10 min and after cooling down to room temperature, the reaction mixture was precipitated with cold ethanol (3 vol.) and centrifuged. Thus, the precipitates contained the enzyme resistant polymers remaining after 5 h (SFSCK-GAL5, 80 mg) and 10 h (SFSCK-GAL10, 77 mg) of incubation and the supernatants contained the fragments released by the enzymatic hydrolysis at 5 h (SFSCK-GAL5S, 20 mg) and 10 h (SFSCK-GAL10S, 23 mg) of incubation.

3 Results and discussion

Fractions CW and CK were obtained from ripe fruits of *A. carambola* employing water and aq. 10% NaOH at 100ºC, respectively. Fraction CK was chosen for further purification due its GPC elution profile, which demonstrated

the presence of fewer peaks than fraction CW (data not shown). Monosaccharide analysis of the fraction CK indicated arabinose as main neutral monosaccharide (Table 1), together with galactose, xylose, glucose and rhamnose. The content of uronic acid was not determined.

A freeze-thaw treatment was applied in fraction CK, giving cold-water soluble polysaccharides (SCK, 3.5% yield) and cold-water insoluble polysaccharides (PCK, 1.4% yield). Monosaccharide analysis demonstrated that fraction SCK (Table 1) has similar composition with CK, having arabinose as major neutral monosaccharide and 5.0% of uronic acids.

The ^{13}C-NMR spectrum of SCK can be seen in Fig. 2A and showed signals at δ 108.1 and δ 107.5 corresponding to anomeric carbons of α-L-Ara*f* units and a signal at δ 104.3 corresponding to β-D-Gal*p* units. These assignments could suggest the presence of an pectic arabinogalactan (Cipriani et al., 2004; Thude & Classen, 2005; Cipriani et al., 2009; Xu, Dong, Qiu, Cong & Ding, 2010; Cordeiro et al., 2012). Although SCK contained 5% of uronic acid, no signal was observed for the carboxyl groups, due to spectral conditions. Moreover, the spectrum also showed anomeric signals of small intensity between δ 98.3-103.2, indicative the presence of another polysaccharide, present in small amount. This mixture was also observed in the analysis of gel permeation chromatography (GPC) of this fraction, which showed a heterogeneous elution profile (Fig. 3A).

In order to purify the arabinogalactan (AG), fraction SCK was submitted to Fehling precipitation (Fig. 1), once arabinogalactans do not complex with Cu^{++} and remain soluble in this treatment (Cantu-Jungles et al., 2014). This strategy was highly efficient, as it produced one homogeneous fraction (SFSCK, 1.9% yield), as could be seen by its elution profile on GPC analysis (Fig. 3A). Its molecular weight was 44kDa. This fraction showed no absorption at 280nm, indicating that it was free of protein. Moreover, it could be seen by monosaccharide analysis that all the xylose, glucose and fucose present in fraction SCK appeared in the Fehling precipitate fraction (PFSCK). PFSCK was composed by fucose, arabinose, xylose, galactose and glucose as neutral sugars (Table 1) and 6.8% of uronic acids.

Monosaccharide analysis of fraction SFSCK showed rhamnose, arabinose, galactose and uronic acid (Table 1). In order to confirm the identity of the uronic acid present in SFSCK, an aliquot of the sample was carboxy-reduced and submitted to monosaccharide analysis by GC-MS. In this procedure, the uronic acid is converted to its corresponding neutral sugar. It was observed an increase in the percentage of galactose in the GC-MS analysis of carboxy-reduced SFSCK indicating that the uronic acid present is the galacturonic acid. The presence of rhamnose and galacturonic acid probably suggested that the AG present in fraction SFSCK is linked to type I rhamnogalacturonan backbone.

The ^{13}C- NMR spectrum of SFSCK is showed in the Fig. 2B and demonstrated the presence of typical signals of α-L-arabinofuranosyl units, with C-1 signals at δ 108.0, δ 107.6 and δ 107.2. Signals of the (1→4)-linked β-D-Gal*p* could be seen at δ 104.3 (C-1), δ 77.6 (substituted C-4), δ 74.5 (C-5), δ 73.5 (C-3), δ 72.0 (C-2) and δ 60.9 (C-6). These assignments are in agreement with published literature data (Cipriani et al., 2004; Thude & Classen, 2005; Cipriani et al., 2009; Xu, Dong, Qiu, Cong & Ding, 2010; Cordeiro et al., 2012). Furthermore, it can be observed in the spectrum, signals compatible with a rhamnogalacturonan structure (RG-I), with C-1 signals at δ 98.3 and δ 97.7 from α-L-Rha*p* and α-D-Gal*p*A units, respectively, and the presence of C-6 of Rha*p* units at δ 16.6 (Colquhoun, Ruiter, Schols & Voragen, 1990; Renard, Lahaye, Mutter, Voragen & Thibault, 1998). Although SFSCK contained 10.4% of galacturonic acid, the signal at δ 174.0-175.0 for carboxy groups did not appear, due to spectral conditions.

Methylation analysis of SFSCK (Table 2) was performed in the carboxy-reduced sample due to the presence of galacturonic acid. The nonreducing terminals consisted of Ara*f* (10.8%) and a small amount of Gal*p* (3.5%). The main observed derivatives were those of arabinose, which was in agreement with monosaccharide analysis. It could be seen in Table 2 the presence of high amounts of 2,3-Me_2-Ara-ol acetate, indicating the presence of (1→5)-linked Ara*f* units. The presence of 3-Me- and 2-Me-Ara-ol acetates indicated that this arabinan was branched at *O*-2 and *O*-3, respectively. The derivatives 3,5-Me_2-

and 2,5-Me_2-Ara-ol acetates indicated (1→2)- and (1→3)-linked Ara*f* units, respectively, probably present as side chains. Arabinopyranose 2-*O*-substituted was also observed in small amounts (1.8%). The methylated derivatives of galactose were 2,3,6-Me_3- and 2,3-Me_2-Gal-ol acetates, wich correspond to (1→4)- and (1→4,6)- linked Gal*p* units. These data indicate a (1→4)-linked galactan as main chain, carrying branches exclusively at *O*-6, suggesting the presence of type I arabinogalactan (AG-I).

This was also observed for arabinogalactans extracted from leaves of *M*. *ilicifolia* (Cipriani et al., 2004), from caja fruits (Iacomini et al., 2005) and pulp of tamarillo fruits (Nascimento et al., 2015). The derivative 3,4-Me_2-Rha-ol acetate demonstrated the presence of (1→2)-linked Rha*p* units. Moreover, the 3-Me-Rha-ol acetate indicated the presence of 2,4-di-*O*-substituted Rha*p* units, which are the insertion point of AG-I in the rhamnogalacturonan backbone. Comparing the methylation analysis of native (data not show) and carboxy-reduced SFSCK, it was possible to observed the increase of the 2,3,6-Me_3-Gal-ol acetate of the carboxy-reduced in relation to native sample, indicating that (1→4)-linked Gal*p*A units were also present. The presence of these units together with (1→2)- and (1→2,4)-linked Rha*p* confirms the presence of type I rhamnogalacturonan.

In order to estimate the relative importance of the neutral side chains to the rhamnogalacturonan backbone, the ratio of (Ara + Gal) to Rha was employed. The lower this ratio, the shorter the side chains attached to 4-*O*-Rha in RG-I backbone (Renard & Ginies, 2009). The calculated ratio was 16.9, demonstrating that SFSCK contained large neutral side chains. Among these neutral side chains, the arabinose to galactose ratio is an estimation of the proportions of Ara versus Gal-rich side chains (Renard & Ginies, 2009). This ratio was 6.0, indicating large amounts of arabinose. To verify the hypothesis that this high arabinose content could arise from a simultaneous presence of an arabinan and a type I arabinogalactan (or galactan) as neutral side chains of the rhamnogalacturonan, an enzymatic degradation was employed. Thus, fraction SFSCK was hydrolyzed with an endo-β-(1→4)-D-galactanase, at two incubation times (5 h and 10 h), followed by ethanol precipitation. The precipitates

(fractions SFSCK-GAL5 and SFSCK-GAL10) showed identical GPC elution profiles and that of fraction SFSCK-GAL5 is shown in Fig. 3B. In comparison with native SFSCK, it demonstrated the presence of a second peak eluted around 60 min. This peak was very intense (Fig. 3B) in the ethanol supernatants (fractions SFSCK-GAL5S and SFSCK-GAL10S) and corresponding to the fragments released by the galactanase treatment.

Monosaccharide analysis of the enzyme resistant polymers (SFSCK-GAL5 and SFSCK-GAL10) also showed similar composition (Table 1), indicating that 5 h of incubation was enough for a complete hydrolysis of the (1→4)-galactan moiety. When compared with SFSCK, both SFSCK-GAL5 and SFSCK-GAL10 fractions demonstrated an increase in their arabinose as well as a decrease in their galactose content. The presence of the small amount of galactose in the enzyme resistant polymers could be due to the presence of (1→6)-linked Gal*p* units and/or resistant short (1→4)-galactooligomers that were not recognized by the enzyme and remained linked to the rhamnogalacturonan backbone, as could be seen in the methylation analysis of SFSCK-GAL5 (Table 2). In agreement with monosaccharide composition, this analysis was predominated by the arabinose derivatives and indicated an (1→5)-linked arabinan branched only at O-3. The side chains were formed by (1→2)- and (1→3)-linked Ara*f* units. Rhamnose (1→2)- and (1→2,4)-linked were also present and arise from the rhamnogalacturonan I backbone. GalA derivatives were not observed, once the sample was not carboxy-reduced prior to the methylation experiment. ^{13}C-NMR spectra (Fig. 4A and B) also demonstrated intense α-L-arabinofuranosyl signals: at δ 107.5 and 107.2 corresponding to anomeric carbons, at δ 83.9-76.2 corresponding to ring carbons and at δ 66.9 and 61.2 corresponding to substituted and free C-5 carbons, respectively. Signals at δ 98.4 and δ 16.6 were also present and arise from C-1 and C-6 of α-L-Rha*p* units, respectively, while small signal at δ 103.4 arise from C-1 of residual β-D-Gal*p* units (Colquhoun et al., 1990; Renard et al., 1998, Cipriani et al., 2004; Thude & Classen, 2005; Cipriani et al., 2009; Xu et al., 2010; Cordeiro et al., 2012). Thus, the above results indicated the presence

of an arabinan-rhamnogalacturonan complex in the galactanase-resistant polymers.

Monosaccharide analysis was performed in the fragments released by the enzymatic hydrolysis at 5 h (SFSCK-GAL5S). It showed the presence of arabinose and galactose (Table 1), indicating the existence of the type I arabinogalactan. Derivatives corresponding to AG-I backbone were observed in the methylation analysis (Table 2), being (1→4)- and (1→4,6)-linked Gal*p* units. The side chains were formed by (1→5)-, (1→3,5)- and (1→3)-linked Ara*f* units. Interestingly, (1→2)-linked Ara*f* units were not observed, being present only in the arabinan moiety of SFSCK-GAL5 fraction. The ^{13}C-NMR spectrum of SFSCK-GAL5S (Fig. 4C) showed anomeric signals at δ 107.5 and δ 104.3 corresponding to α-L-Ara*f* and β-D-Gal*p* units, respectively. The signals at δ 96.4 and 92.4 corresponding to the β- and α- anomeric carbons, respectively, of galactose units in the reducing-end (Gorin & Mazurek, 1975).

Finally, the enzymatic degradation results demonstrated that fraction SFSCK contains a pectin, formed by a rhamnogalacturonan I to which a branched arabinan and a type I arabinogalactan are attached. The proportion of arabinan side chain is higher than arabinogalactan. In fruits, RG-I with side chains richer in arabinans than in galactans were already found in albedo cell walls of orange (*Citrus sinensis*) (Prabasari et al., 2011) and in *Argania spinosa* fruit pulp (Aboughe-Angone et al., 2008). Moreover, RG-I with similar amounts of Ara and Gal were extracted from citrus peel (Yapo et al., 2007), while RG-I branched with type I arabinogalactans was purified from caja fruits and prunes (Iacomini et al., 2005, Cantu-Jungles et al., 2014). A highly ramified RG-I associated with cellulose was characterized in ripe apples (Oechslin et al., 2003). Attached to its backbone was a xylogalacturonan, a highly ramified arabinan, a (1→4)-linked galactan with few (1→3)-and (1→6)-linked short chains and a short strictly linear (1→4)-galactooligomers.

4 Conclusions

A pectin, formed by a rhamnogalacturonan I to which a branched arabinan and a type I arabinogalactan are attached was purified and characterized from starfruit. It was composed of rhamnose, arabinose, galactose and uronic acid in the 5.0:72.5:12.1:10.4 molar ratios, respectively. After enzymatic degradation procedure with an endo-β-(1→4)-D-galactanase, an arabinan-rhamnogalacturonan remained as the resistant polymer, while the arabinogalactan fragments were released.

Acknowledgements

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TABLE 1 – MONOSACCHARIDE COMPOSITION OF FRACTIONS OBTAINED FROM THE FRUIT OF STARFRUIT (*Averrhoa carambola* L.)

Fractions	Monosaccharide composition (%)[a]						
	Rha	Fuc	Ara	Xyl	Gal	Glc	Uronic acid[b]
CK	1.4	-	77.5	7.2	8.7	5.2	nd[c]
SCK	1.7	tr[d]	69.1	7.4	8.7	8.0	5.0
PFSCK	-	2.3	10.2	37.8	9.6	33.3	6.8
SFSCK	5.0	-	72.5	-	12.1	-	10.4
SFSCK-GAL5	5.0	-	83.6	-	4.4	-	7.0
SFSCK-GAL10	4.5	-	83.3	-	5.2	-	7.0
SFSCK-GAL5S	-	-	70.3	-	29.7	-	-

[a] % of peak area relative to total peak areas, determined by GC–MS.

[b] Determined spectrofotometrically using the *m*-hydroxybiphenyl method (Filisetti-Cozzi & Carpita, 1991).

[c] Not determined.

[d] Trace amounts.

TABLE 2 – LINKAGE TYPES BASED ON ANALYSIS OF PARTIALLY *O*-METHYL ALDITOL ACETATES OF FRACTIONS SFSCK, SFSCK-GAL5 AND SFSCK-GAL5S

Partially *O*-methylalditol acetate	**Linkage type[b]**	**SFSCK[c]**	**SFSCK-GAL5**	**SFSCK-GAL5S**
2,3,5-Me_3-Ara[a]	Ara*f*-(1→	10.8	10.8	11.9
3,5-Me_2-Ara	→2)-Ara*f*-(1→	8.3	12.4	-
2,5-Me_2-Ara	→3)-Ara*f*-(1→	7.8	8.0	7.3
2,3-Me_2-Ara	→5)-Ara*f*-(1→	35.7	37.3	34.8
3,4-Me_2-Ara	→2)-Ara*p*-(1→	1.8	-	-
2-Me-Ara	→3,5)-Ara*f*-(1→	9.3	15.1	8.2
3-Me-Ara	→2,5)-Ara*f*-(1→	1.1	-	-
2,3,4,6-Me_4-Gal	Gal*p*-(1→	3.5	6.7	27.0
2,3,6-Me_3-Gal	→4)-Gal*p*-(1→	13.0[d]	2.2	8.5
2,3,4-Me_3-Gal	→6)-Gal*p*-(1→	1.1	0.9	-
2,3-Me_2-Gal	→4,6)-Gal*p*-(1→	1.7	-	2.3
2,3,4-Me_3-Rha	Rha*p*-(1→	1.1	-	-
3,4-Me_2-Rha	→2)-Rha*p*-(1→	3.8	3.4	-
3-Me-Rha	→2,4)-Rha*p*-(1→	1.2	3.4	-

[a] 2,3,5-Me_3-Ara = 2,3,5-tri-*O*-Methylarabinitolacetate, etc. [b] Based on derived *O*-methylalditol acetates. [c] % of peak area of *O*-methylalditol acetates relative to total area, determined by GC-MS. Samples were carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972), prior to methylation analysis. [d] Also includes the percentage of carboxy-reduced Gal*p*A (10.4%).

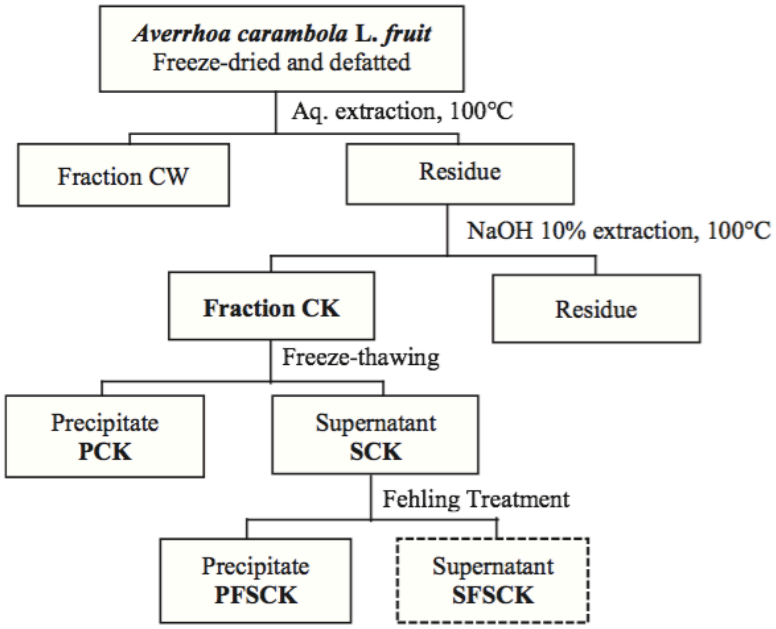

FIGURE 1 – SCHEME OF EXTRACTION AND FRACTIONATION OF FRACTION SFSCK FROM THE FRUIT OF STARFRUIT (*Averrhoa carambola* L.)

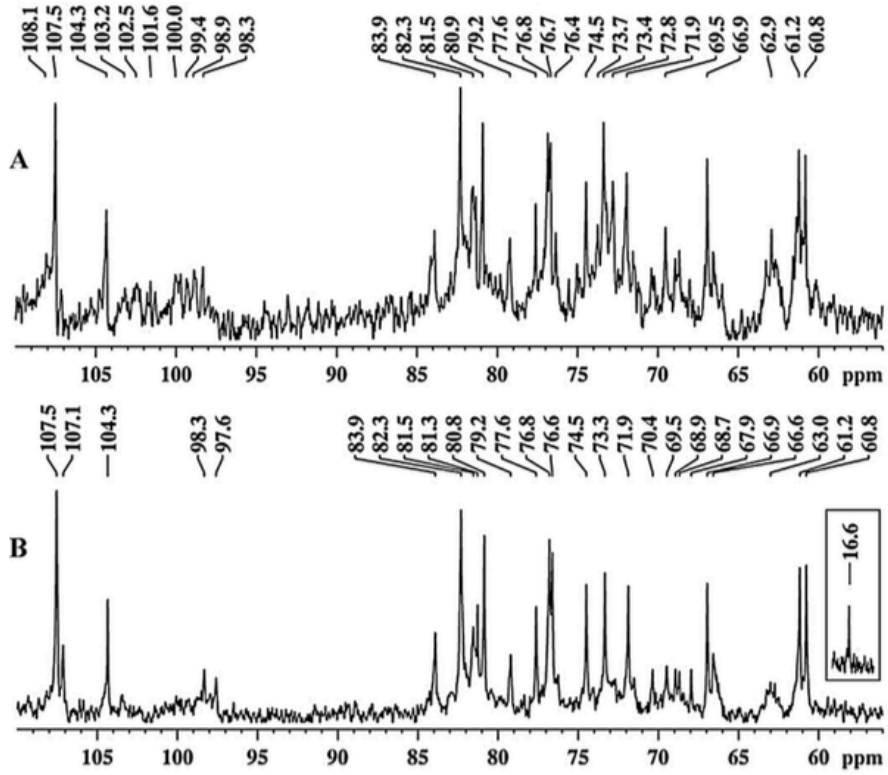

FIGURE 2 – ^{13}C-NMR SPECTRA OF FRACTION SCK (A) AND FRACTION SFSCK (B), IN D_2O AT 50ºC

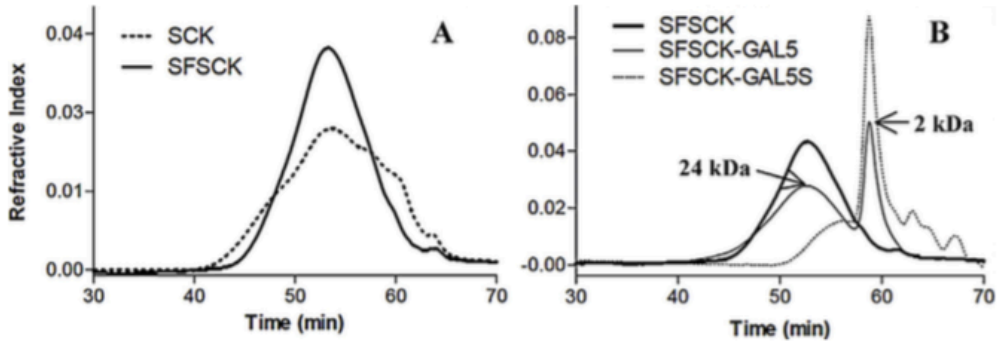

FIGURE 3 – GPC ELUTION PROFILE OF (A) FRACTIONS SCK AND SFSCK AND (B) FRACTION SFSCK BEFORE AND AFTER INCUBATION WITH ENDO-GALACTANASE (FRACTIONS SFSCK-GAL5 AND SFSCK-GAL5S). REFRACTIVE INDEX DETECTOR

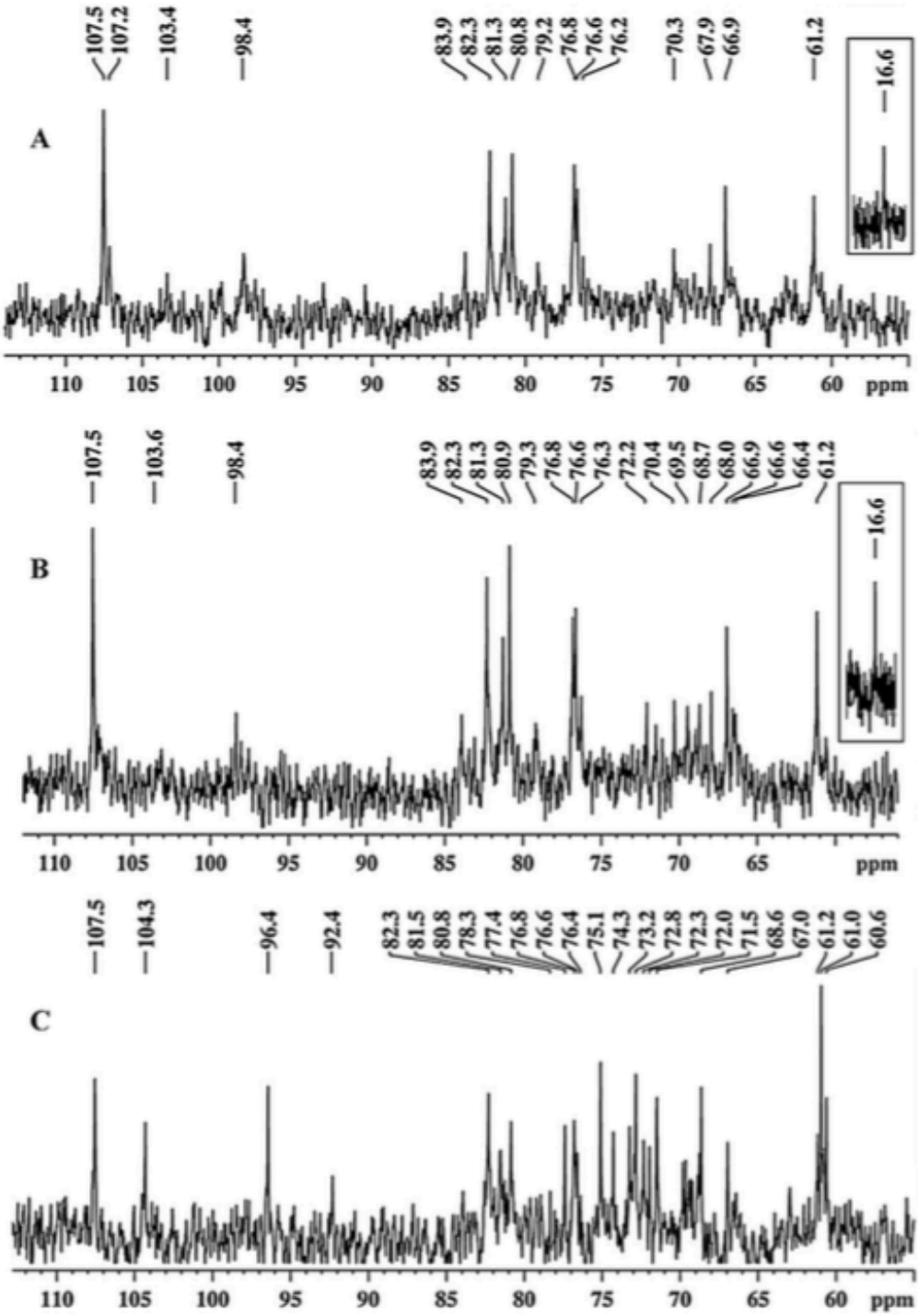

FIGURE 4 – ^{13}C-NMR SPECTRA OF (A) FRACTION SFSCK-GAL5, (B) FRACTION SFSCK-GAL10 AND (C) FRACTION SFSCK-GAL5S, IN D_2O AT 50ºC

ARTIGO II

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Substituted galacturonan from starfruit: chemical structure and antinociceptive and anti-inflammatory effects

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ABSTRACT

Starfruit (*Averrhoa carambola* L.) is an edible tropical fruit, which is usually consumed as a fresh table fruit or as fruit juice. It also exhibits various pharmacological activities. In this study, polysaccharides were extracted with boiling water and purified by freeze-thawing and Fehling treatments. After purification steps, a homogenous fraction was obtained. It was analyzed by sugar composition, gel permeation chromatography, methylation, and two-dimensional nuclear magnetic resonance (2D NMR) spectroscopy analyses. It comprised arabinose (Ara), galactose (Gal), and galacturonic acid (GalA) in a molar ratio of 12.3:1.7:86.0. Methylation and NMR spectroscopy analyses showed that it contained a substituted galacturonan composed of (1→4)-linked *α*-D-Gal*p*A units branched at *O*-2 by (1→5)-linked *α*-L-Ara*f* and terminal *α*-L-Ara*f* and *α*-D-Gal*p*A units. The effect of PFSCW (10–300 mg/kg, i.p.) on nocifensive behavior induced by intraplantar injection of formalin in mice was evaluated. The fraction demonstrated antinociceptive and anti-inflammatory properties, suggesting that it may be useful in therapeutic intervention for the management of inflammatory pain.

Keywords: Starfruit; substituted galacturonans; anti-inflammatory and antinociceptive activities.

1. Introduction

Inflammation has been recognized as a major risk factor for various progressive diseases in humans, including cancer, neurological disease, metabolic disorders, and cardiovascular disease, and targeting the reduction of chronic inflammation is a beneficial strategy to prevent these diseases [1]. Dietary fibers are plant cell wall polysaccharides, and the intake of these molecules is inversely associated with inflammatory markers [2]. Among these polysaccharides are pectins. Pectins are a family of complex polysaccharides that contain (1→4)-linked α-D-Gal*p*A residues [3]. Homogalacturonan (HG); rhamnogalacturonan I; and substituted galacturonans such as rhamnogalacturonan II, xylogalacturonan, apiogalacturonan, galactogalacturonan, arabinogalacturonan, and galacturonogalacturonan are the pectic polysaccharides that have been found in primary cell walls [4]. Anti-inflammatory activity has also been demonstrated by purified pectins, such as for those found in sweet pepper, celery stalks, *Comarum palustre*, and *Potamogeton natans* [5-8].

Averrhoa carambola L., also known as starfruit or carambola, is an edible tropical fruit of the Oxalidaceae family, usually consumed as fresh fruit or as fruit juice [9-11]. Several studies have reported that different parts of the *A. carambola* tree exhibit pharmacological activities [12]. In the fruits, antioxidant [13] and hypoglycemic effects [14,15] have been reported, while anti-inflammatory effects have been described for leaves [16] and stems [17]. Thus far, the biological activities of the polysaccharides of starfruit have not been reported. Recently, Leivas et al. [18] described a pectin from starfruit, formed by a type I rhamnogalacturonan containing arabinan and type I arabinogalactan as the neutral side chains. Herein, we describe the chemical structure of a substituted galacturonan extracted from *A. carambola* fruits and report the findings on its antinociceptive and anti-inflammatory effects.

2. Materials and methods

2.1. Plant material

Ripe starfruits of the cultivar B10 were purchased from the local market of Curitiba, State of Paraná, Brazil.

2.2. General analytical methods

All solutions were evaporated below 60°C under reduced pressure. Centrifugation was performed at 12,000 ×*g* for 20 min at 10°C. Dialysis was performed using a 6–8 kDa molecular weight cut-off membrane (Spectra/Por®). Total lipid quantification was performed by extraction employing chloroform-methanol (1:1) as a solvent through the Soxhlet apparatus.

Uronic acid (UA) contents were determined using the *m*-hydroxybiphenyl colorimetric method [19] using galacturonic acid as the standard. Fraction was carboxy-reduced by the carbodiimide method [20] using $NaBH_4$ as the reducing agent, yielding products with the –COOH groups of its UA residues reduced to –CH_2OH.

The degree of methylesterification (DE) was determined by ^1H nuclear magnetic resonance (NMR) spectroscopy [21] using a Bruker AVANCE III 400 NMR spectrometer.

2.3. Extraction and purification of polysaccharides

Polysaccharides from starfruit were extracted with boiling water, yielding the aqueous fraction (CW) (Fig. 1) as previously described by Leivas et al. [18]. A freeze-thaw treatment was applied in this fraction to yield cold-water soluble (SCW) and insoluble (PCW) fractions. In this procedure, the sample was frozen and then thawed at room temperature, followed by centrifugation, until no more precipitate appeared.

Fraction SCW was dissolved in distilled water and then treated with Fehling's solutions [22], resulting in soluble (SFSCW) and precipitated (PFSCW) fractions,

which were separated by centrifugation. Each fraction was neutralized with acetic acid (HOAc), dialyzed against tap water, and deionized with cation exchange resin.

Polysaccharide yields were expressed as percentages based on the weight of dried starfruit subjected to extraction (310.7 g).

2.4. Monosaccharide analysis

Neutral sugars were hydrolyzed with 2 M trifluoroacetic acid for 8 h at 100°C, reduced with $NaBH_4$ [23], and acetylated with acetic anhydride-pyridine (1:1, v/v) for 18 h at 25°C [24]. The resulting alditol acetates were analyzed by gas chromatography mass spectrometry (GC-MS) following the conditions employed by Leivas et al. [18].

2.5. Determination of homogeneity and molecular weight of polysaccharides

The homogeneity and molecular weight of water-soluble polysaccharides were determined by gel permeation chromatography (GPC). The procedure was carried out as previously reported by Leivas et al. [18].

2.6. Methylation analysis of polysaccharides

Fraction PFSCW was carboxy-reduced by the carbodiimide method [20] and methylated according to Ciucanu and Kerek [25] using powdered NaOH in DMSO-MeI. The per-*O*-methylated polysaccharide was then subjected to methanolysis in 3% HCl–MeOH (at 80°C, 2 h), followed by hydrolysis with H_2SO_4 (0.5 M, 10 h, at 100°C) and neutralization with $BaCO_3$. The resulting mixture of partially *O*-methylated monosaccharides was successively reduced with $NaBD_4$ and acetylated with acetic anhydride-pyridine. The products (partially *O*-methylated alditol acetates) were examined by capillary GC-MS. A capillary column (30 m × 0.25 mm i.d.) of DB-225, held at 50°C during injection for 1 min, programmed at 40°C/min to 210°C, and held at this temperature for 31 min was used for separation. The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times [26].

2.7. Nuclear magnetic resonance spectroscopy

^{13}C NMR and 2D $^{1}H/^{13}C$ heteronuclear single quantum coherence spectroscopy (HSQC) spectra were acquired at 50°C on a Bruker AVANCE III 400 NMR spectrometer operating at 9.5 T, observing ^{1}H at 400.13 MHz and ^{13}C at 100.61 MHz, and equipped with a 5-mm multinuclear inverse detection probe with z-gradient. Samples were dissolved in D_2O, and chemical shifts were expressed as δ ppm relative to the CH_3 signal from acetone at δ 30.2 as the internal reference. ^{1}H NMR spectra were acquired at 70°C, with 256 scans on a Bruker AVANCE III 400 NMR spectrometer. Samples were deuterium-exchanged 3 times by freeze-drying with D_2O solutions, finally dissolved in D_2O, and transferred into 5-mm NMR tubes. Chemical shifts were expressed as δ ppm relative to HDO at δ 4.22 as the internal reference.

2.8. Experimental animals

Experiments were conducted using adult female Swiss mice (25–35 g), obtained from the animal facility of Universidade Federal de Santa Catarina (UFSC, Florianópolis, SC, Brazil). Animals were housed in cages under a 12 h:12 h light:dark cycle (lights on at 6:00 am) at a controlled temperature (22°C ± 2°C) with *ad libitum* access to food and water. The animals were homogeneously distributed among groups and acclimatized in the experimental room for at least 1 h before testing and were used only once throughout the experiments. The experiments were performed after approval from the Ethics Committee for Animal Research of Universidade Federal de Santa Catarina (CEUA/UFSC protocol number PP00745) in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals [27]. The number of animals used and the intensity of the noxious stimuli were at the necessary minimum to demonstrate the consistent effects of drug treatments.

2.9. Drugs

The following substances were used: formaldehyde, Tween 80 (Merck, Darmstadt, Germany); meloxicam (Movatec®, Boehringer Ingelheim, São Paulo,

Brazil), indomethacin, diclofenac (Sigma Chemical Co., St. Louis, MO, USA), and acetaminophen. Meloxicam and diclofenac were dissolved in 0.5% Tween 80 plus 0.9% (w/v) NaCl solution; indomethacin was dissolved in saline with 5% sodium carbonate. The final concentration of Tween 80 and sodium carbonate did not exceed 5% and did not cause any effect *per se*.

2.10. Formalin test

The applied procedure was similar to that described in other studies [28,29]. After an adaptation period and treatment, mice were administered 20 µL of a 2.5% formalin solution (0.92% formaldehyde) in saline via an intraplantar injection in the ventral surface of the right hind paw. After formalin injection, the animals were immediately placed individually in an acrylic chamber, and the time spent licking the injected paw was recorded with a chronometer for both the early neurogenic phase (0–5 min) and late inflammatory phase (15–30 min) of this model. Time spent licking the injected paw was recorded as an indicator of nociception. Mice were treated intraperitoneally (i.p.) with PFSCW (10–300 mg/kg) or vehicle (10 mL/kg) 0.5 h before formalin injection. The positive control groups were pre-treated with indomethacin (10 mg/kg), meloxicam (10 mg/kg), diclofenac (30 mg/kg), or acetaminophen (100 mg/kg) 0.5 h before formalin injection. In addition, paw edema of the animals was verified before and after the observation time of the nociceptive response induced by intraplantar injection of formalin. The thickness difference (Δ paw thickness, in millimeters) between the right paw before and after formalin injection, was considered an index of edema, measured from the central region of the paw using an electronic digital micrometer (0–25 mm; MT-045B; Shanghai Metal Great Tools Co., Shanghai, China).

3. Results and discussion

3.1. Structural characterization of galacturonan from starfruit

Polysaccharides were extracted from ripe fruits of *A. carambola* using boiling water to yield fraction CW (7% yield). Its monosaccharide analysis indicated

Ara:Glc:Gal:Xyl:Rha as neutral sugars in a 76.0:10.5:7.9:3.2:2.4 molar ratio. UA content was not determined. A freeze-thaw treatment was then applied, yielding SCW (yield, 4.3%) and PCW (yield, 0.6%) polysaccharides.

Monosaccharide analysis of SCW revealed Ara:Gal:Xyl:Rha as neutral sugars in a 9.0:2.4:1.8:1.6 molar ratio and 85.2% UAs, indicating the presence of pectic polysaccharides. Its ^{13}C NMR spectrum (Fig. 2) showed anomeric signals at *δ* 109.1, 107.6, and 107.2 corresponding to C-1 from *α*-L-Ara*f* units and signals at *δ* 104.3, 103.4, and 103.2, which can be attributed to C-1 from *β*-D-Gal*p* units. These assignments could suggest the presence of a pectic arabinogalactan (AG), similar to that previously reported for starfruit [18]. Furthermore, signals at *δ* 100.1 and 99.3, corresponding to anomeric carbons of esterified and unesterified units of *α*-D-Gal*p*A, respectively, could be observed in the spectrum. Their respective C-6 signals were seen at *δ* 170.7 and 174.8, from methyl ester carbonyl carbons and carboxyl carbons, respectively. The signal at *δ* 52.8 could be attributed to methyl carbons of esterified carbonyls in Gal*p*A units. These assignments are in agreement with published literature data [30-33] and probably indicate the presence of a methyl esterified homogalacturonan (HG). GPC analysis demonstrated the presence of a broad peak (Fig. 3).

Due to the presence of methylesterified *α*-D-Gal*p*A units, the DE was determined by ^{1}H NMR spectroscopy [21]. A value of 80% was found for SCW, suggesting a classification as a high-methoxyl (HM) pectin [34].

For the separation of the HG from the AG, fraction SCW was subjected to Fehling precipitation [22] (Fig. 1). The HG formed a complex with Cu^{++} and precipitated (fraction PFSCW), while AG did not complex with Cu^{++} and remained soluble (fraction SFSCW) in this treatment [35]. This strategy was highly efficient, as it produced one homogenous fraction (PFSCW, 2.8% yield), as could be seen by its elution profile on GPC analysis (Fig. 3). Its molecular weight was 40 kDa. This fraction showed no absorption at 280 nm, indicating that it was free of protein.

Due to the presence of UAs, monosaccharide analysis of PFSCW was performed on native and carboxy-reduced samples, demonstrating Ara:Gal:UA in 12.3:1.7:86.0 and Ara:Gal in 12.0:88.0 molar ratios, respectively. To achieve the linkages present in this pectic polysaccharide, the fraction was carboxy-reduced and subjected to methylation analysis (Table 1). In agreement with monosaccharide

composition, the main observed derivatives were those of galactose, which arose from Gal*p*A units. As is evident from Table 1, the presence of high amounts of 2,3,6-Me_3-Gal-ol acetate indicated (1→4)-linked Gal*p*A units. The presence of 3,6-Me_2-Gal-ol acetate indicated that this galacturonan was branched at *O*-2. The presence of 2,3-Me_2-Ara-ol acetate indicated (1→5)-linked Ara*f* units. Gal*p*A and a small amount of Ara*f* were also found as terminal units. Fraction PFSCW was also analyzed by NMR analysis. Due to the alkaline pH employed in the Fehling treatment, PFSCW resulted in an unesterified polysaccharide [36]. HSQC results (Fig. 4) showed signals at *δ* 99.0/5.09 (C1/H1), 68.3/3.75 (C2/H2), 68.7/3.98 (C3/H3), 77.9/4.43 (C4/H4), and 71.2/4.76 (C5/H5), corresponding to unesterified (1→4)-*α*-D-GalpA units. Due to de-esterification, the signal at *δ* 52.8 (Fig. 2) attributed to methyl carbons of esterified carbonyls in Gal*p*A units disappeared, while the signal at *δ* 170.7 assigned to carboxyl groups bound by methyl groups shifted to *δ* 175.0 (seen in the ^{13}C NMR spectrum, data not shown). Moreover, HSQC (Fig. 4) also showed *α*-L-arabinofuranosyl signals at δ 107.5/5.08 (C1/H1), *δ* 66.7/3.88 (C5/H5), and *δ* 66.4/3.81 (C5'/H5'), corresponding to (1→5)-linked units, and signals at *δ* 107.0/5.15 (C1/H1), *δ* 61.0/3.82 (C5/H5), and *δ* 61.0/3.73 (C5'/H5'), corresponding to terminal units [31, 32, 36, 37]. Therefore, these results indicated that PFSCW contains a substituted galacturonan composed of (1→4)-linked *α*-D-Gal*p*A units branched at *O*-2 by (1→5)-linked *α*-L-Ara*f* and terminal *α*-L-Ara*f* and *α*-D-Gal*p*A units.

Substituted galacturonans have been reported in the literature, such as rhamnogalacturonan II, xylogalacturonan, apiogalacturonan, galactogalacturonan, arabinogalacturonan, and galacturonogalacturonan [4]. Substituted galacturonans that also have *α*-L-Ara*f* and *α*-D-Gal*p*A units in the side chains, as observed herein for *A. carambola*, have been found in apple [39] and *Comarum palustre* [40].

3.2. Antinociceptive and anti-inflammatory activities

Intraplantar injection of formalin displayed a typical biphasic nocifensive behavior. Mice spent approximately 80 s displaying nociceptive behaviors during the first 5 min of the assay (neurogenic pain, phase 1), and approximately 300 s during the period of 15–30 min (inflammatory pain, phase 2) (Fig. 5A and B). In addition, formalin induced marked paw edema (~1 mm thick), which is associated with the

release of inflammatory mediators present in phase 2 of this model (Fig. 5C). The results depicted in Figure 5 (A and B) show that intraperitoneal administration of PFSCW produced a graded and significant inhibition of the inflammatory (15–30 min) phase, and, to a lesser extent, the neurogenic (0–5 min) phase of formalin-induced nociception. However, PFSCW was more potent and efficacious in inhibiting the inflammatory than the neurogenic component of the formalin pain response. The calculated mean ID_{50} value and 95% confidence limit for the inflammatory phase was 37.94 (26.36–54.62) mg/kg, and maximal inhibition values were 31% ± 6% and 99% ± 1% for the early and late phase at the doses of 100 and 300 mg/kg, respectively (Fig. 5A and B). Furthermore, PFSCW (100 and 300 mg/kg) also reduced paw edema caused by intraplantar formalin injection when compared to the control group, with inhibition of 53% ± 5% at the dose of 300 mg/kg, suggesting that its antinociceptive effect may be secondary to the anti-inflammatory property.

Of note, electrophysiological and behavioral studies demonstrated that primary nociceptive afferent fibers (Aδ-fibers and C-fibers) exhibit sustained firing during both phases of the formalin test [41-43]. Furthermore, the neurogenic phase of formalin occurs by direct activation of receptors from primary nociceptive neurons, whereas the second phase occurs by combined effects of the release of inflammatory mediators (such as histamine, serotonin, prostaglandins, and kinins) and central sensitization in the dorsal horn [43-46]. Studies emphasize that kinins, prostanoids, histamine, and serotonin, but not tachykinin, play an important modulatory role in controlling formalin-induced edema formation [43, 45-47]. In addition, experimental data showed that non-steroidal anti-inflammatory drugs (NSAIDs) are poorly effective in attenuating the neurogenic component of formalin-induced pain [43-48]. Herein, we extend these previous data and demonstrate that the NSAIDs used as positive controls, such as indomethacin, acetaminophen, meloxicam, and diclofenac, produced significant inhibition of the inflammatory phase (15–30 min) of the formalin model, with inhibition of 64% ± 4%, 88% ± 6%, 82% ± 4%, and 73% ± 8%, respectively (Table 2). While both meloxicam and diclofenac also reduced the neurogenic phase caused by formalin (inhibition of 58% ± 3% and 35% ± 6%, respectively), meloxicam was more efficient (Table 2). However, only meloxicam and indomethacin were able to significantly reduce the paw edema caused by formalin injection compared with the control group, (reduction of 31% ± 6% and 34%

± 6%, respectively) (Table 2). Of note, this reduction in paw edema was similar to that observed with PFSCW (Figure 5C).

Considering that the formalin test is a well-established model for assessing nociceptive processes and analgesic drug effects [49], besides being the current pain model that most closely resembles clinical conditions [50], it has been used in a variety of studies on the effects and function of exogenous and endogenous substances such as opioids [43, 50, 51], monoamines [52], and other compounds [53,54]. Thus, the results of this present study demonstrated, for the first time, the analgesic and anti-inflammatory properties of PFSCW on a formalin model, suggesting that it may be useful in therapeutic intervention for the management of inflammatory pain. How PFSCW induces the analgesic and anti-inflammatory effects still needs to be further clarified.

With regard to the biological activities of pectic polysaccharides composed mainly of galacturonans, anti-inflammatory effects have also been reported for apiuman from *Apium graveolens* [5], comaruman from *Comarum palustre* [6], potamogetonan from *Potamogeton natans* [7], and capsicuman from *Capsicum annum* [8].

4. Conclusions

In this study, we obtained a water-extractable polysaccharide from starfruit. It was characterized as a substituted galacturonan by monosaccharide, GPC, methylation, and 2D NMR analyses. This polysaccharide demonstrated antinociceptive and anti-inflammatory properties in a formalin model, suggesting that it has potential health benefits and may be useful in therapeutic intervention for the management of inflammatory pain.

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TABLE 1 – LINKAGE TYPES BASED ON ANALYSIS OF PARTIALLY *O*-METHYL ALDITOL ACETATES OF FRACTION PFSCW

Partially *O*-methylalditol acetate	**Linkage type[b]**	**mol%[c]**
2,3,5-Me_3-Ara[a]	Ara*f*-(1→	1.7
2,3-Me_2-Ara	→5)-Ara*f*-(1→	5.4
2,3,4,6-Me_4-Gal	Gal*p*-(1→	10.1
2,3,6-Me_3-Gal	→4)-Gal*p*-(1→	68.9
3,6-Me_2-Gal	→2,4)-Gal*p*-(1→	13.9

[a] 2,3,5-Me_3-Ara = 2,3,5-tri-*O*-methylarabinitolacetate, etc.

[b] Based on derived *O*-methylalditol acetates.

[c] According to Pettolino et al. [55]. Samples were carboxy-reduced by the carbodiimide method [20] prior to methylation analysis.

TABLE 2 – EFFECT OF INDOMETHACIN (10 mg/kg, I.P.), MELOXICAM (10 mg/kg, I.P.), DICLOFENAC (30 mg/kg, I.P.), OR ACETAMINOPHEN (10 mg/kg, I.P.) ON NOCIFENSIVE BEHAVIOR INDUCED BY INTRAPLANTAR INJECTION OF FORMALIN IN MICE

Drugs	**Nociceptive Response**		**Paw edema**
(i.p.)	**0–5 min**	**15–30 min**	**(mm)**
Control	89.2 ± 7.0	264.2 ± 7.16	0.94 ± 0.07
Indomethacin	89.8 ± 3.5	95.1 ± 7.9**	0.62 ± 0.03**
Meloxicam	37.5 ± 3.9**	47.5 ± 5.8**	0.64 ± 0.03**
Diclofenac	58.0 ± 4.2**	71.4 ± 4.7**	0.82 ± 0.09
Acetaminophen	82.8 ± 6.3	58.7 ± 9.5**	0.89 ± 0.04

The total time spent licking (mean ± SEM) the hind paw was measured during the neurogenic phase (0–5 min) and the inflammatory phase (15–30 min). Paw edema corresponding to the difference between paw thickness before and after formalin challenge. Each value represents the mean value obtained for 6 to 8 animals, and the vertical lines indicate the SEM. The asterisks denote the significance levels when compared with the control group (one-way ANOVA, followed by Student-Newman-Keuls test), **$p < 0.01$.

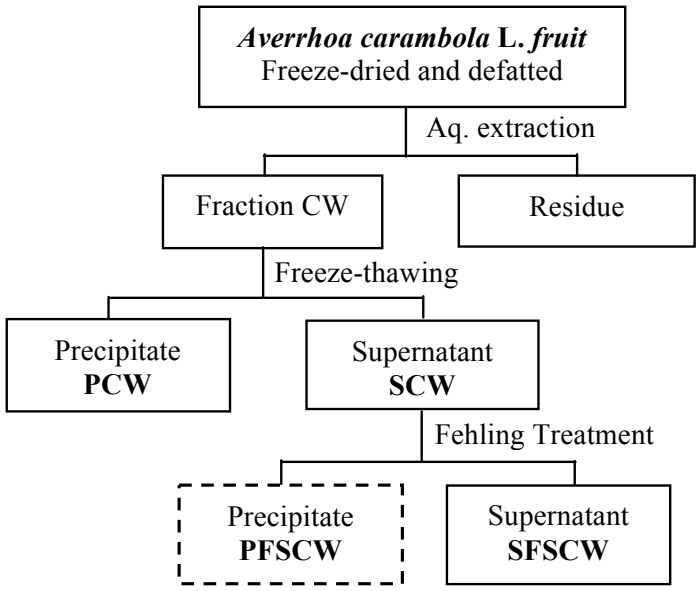

FIGURE 1 – SCHEME OF EXTRACTION AND FRACTIONATION OF A GALACTURONAN FROM STARFRUIT (*Averrhoa carambola* L.)

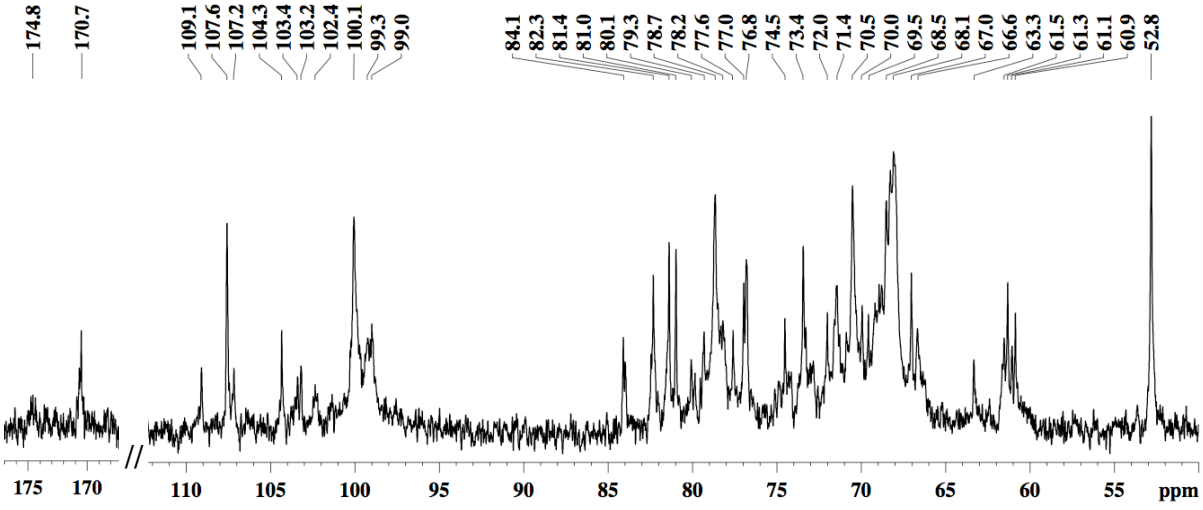

FIGURE 2 – ^{13}C NMR SPECTRUM OF FRACTION SCW IN D_2O AT 50°C

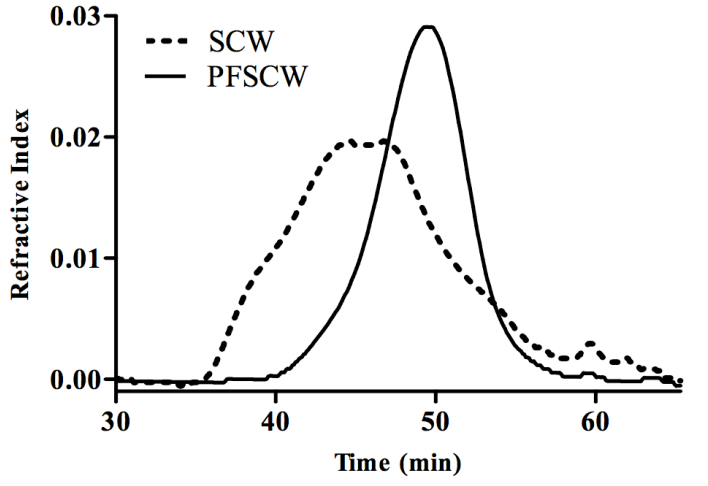

FIGURE 3 – GPC ELUTION PROFILE OF SCW AND PFSCW FRACTIONS OBTAINED USING A REFRACTIVE INDEX DETECTOR

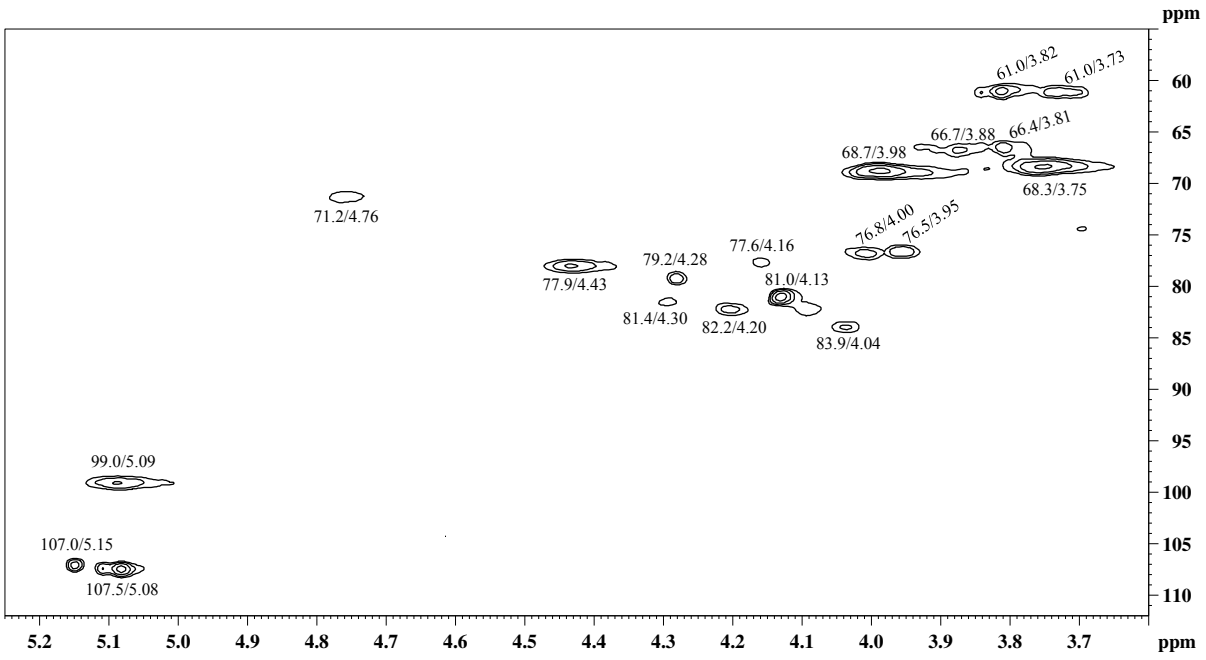

FIGURE 4 – 2D $^{1}H/^{13}C$ HSQC SPECTRUM OF FRACTION PFSCW IN D_2O AT 50°C

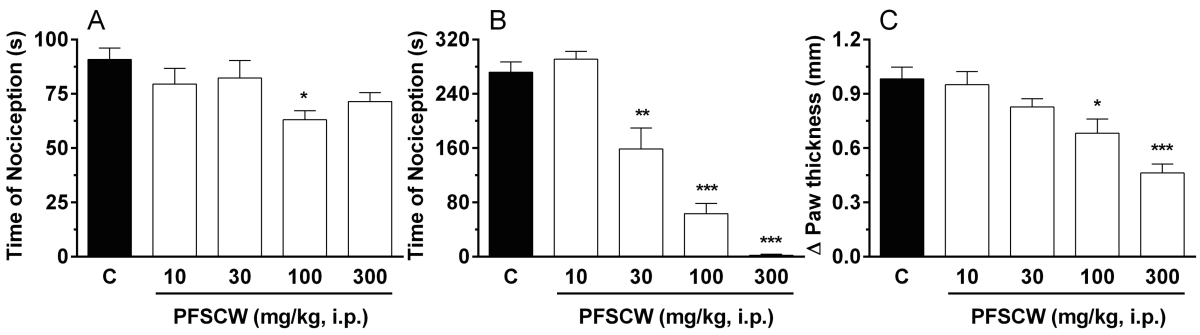

FIGURE 5 – EFFECT OF PFSCW (10–300 mg/kg, I.P.) ON NOCIFENSIVE BEHAVIOR INDUCED BY INTRAPLANTAR INJECTION OF FORMALIN IN MICE. THE TOTAL TIME SPENT LICKING THE HIND PAW WAS MEASURED DURING THE NEUROGENIC PHASE (0–5 min, PANEL A) AND THE INFLAMMATORY PHASE (15–30 min, PANEL B). PANEL C REPRESENTS THE PAW EDEMA CORRESPONDING TO THE DIFFERENCE BETWEEN PAW THICKNESS BEFORE AND AFTER FORMALIN CHALLENGE. EACH COLUMN REPRESENTS THE MEAN VALUE OBTAINED FOR 8–12 ANIMALS, AND THE VERTICAL LINES INDICATE THE SEM. THE ASTERISKS DENOTE THE SIGNIFICANCE LEVELS WHEN COMPARED WITH THE CONTROL GROUP (C, ONE-WAY ANOVA, FOLLOWED BY STUDENT-NEWMAN-KEULS TEST), *P < 0.05, **P < 0.01 AND ***P < 0.001.

ARTIGO III

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Pectic type II arabinogalactans from starfruit (*Averrhoa carambola* L.)

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ABSTRACT

A structural characterization of polysaccharides from edible tropical fruit named starfruit (*Averrhoa carambola* L.*)* was carried out. After the purification steps, two homogenous fractions were obtained. Fraction 50R was composed of rhamnose, arabinose, galactose and uronic acid in 4.3:56.2:37.4:2 molar ratio, respectively and fraction 10R was composed of rhamnose, arabinose, galactose and uronic acid in 2.8:65.8:28.5:3 molar ratio, respectively. Methylation and NMR espectroscopy analysis showed that these fractions are formed by pectic arabinogalactans, which contain (1→3), (1→6) and (1→3,6)-linked Gal*p* units. The side chains have 3-*O*-, 5-*O*- and 3,5-di-*O*-linked α-Ara*f* and nonreducing end-units of α-Ara*f*, Ara*p*, β-Gal*p* and α-Glc*p*A. These arabinogalactans were linked to type I rhamnogalacturonans.

Keywords: Starfruit, *Averrhoa carambola*, pectic type II arabinogalactans.

1 Introduction

Starfruit (*Averrhoa carambola* L.) is an edible tropical fruit of Oxalidaceae family, native of Malaysia and cultivated in various other Asian countries and in tropical areas of America, including Brazil (Manda, Vyas, Pandya, & Singhal, 2012; O'Hare, 1993; Soncini et al., 2011). Pharmacological activities have been reported

for different parts of *A. carambola* tree (Dasgupta, Chakraborty & Bala, 2013), such as fruit, leaves and stems. For fruits, antioxidant (Shui & Leong, 2004) and hypoglycemic effects (Chau, Chen & Lin, 2004; Gunasekara, Fernando & Sivakanesan, 2011) have already been reported.

In Brazil, starfruit is usually consumed as fresh fruit or as juice fruit (Ferreira, Fernandes, Galende, Cortez & Bazotte, 2008) and this fruit is low in fat and calories and has high amounts of carbohydrates (91.3% of their total macronutrients) (Manda et al., 2012; Taco-Unicamp, 2011). Until recently, there were no studies dealing with the identification and characterization of starfruit's polysaccharides. Leivas, Iacomini and Cordeiro (2015) described for the first time the structure of a starfruit's pectin, formed by a type I rhamnogalacturonan containing arabinan and type I arabinogalactan as neutral side chains. Herein we report the isolation, composition and structural features of two fractions containing type II arabinogalactans.

Arabinogalactans are cell wall polysaccharides with high proportions of galactose and arabinose and are widely distributed in the plant kingdom. They occur in different structural types: a) type I arabinogalactans have linear (1→4)-β-D-Gal*p* backbone, b) type II arabinogalactans consist of (1→3)- and (1→6)-linked β-D-Gal*p* chains connected to each other by (1→3, 6)-linked branch point residues (Clarke, Anderson & Stone, 1979) and c) arabinogalactans with (1→6)-linked β-D-Gal*p* backbone branched at C-3 by α-L-arabinosyl side chains have also been described in some plant sources (Capek, 2008; Dong & Fang, 2001; Kiyohara, Yamada & Otsuka, 1987; Oliveira, Cordeiro, Gonçalves, Ceole, Ueda-Nakamura & Iacomini, 2013; Raju & Davidson, 1994; Wagner & Jordan, 1988).

Pectic type II arabinogalactans have been isolated from different parts of plants, mainly in leaves (Cipriani et al., 2006; Dong & Fang, 2001; Duan, Wang, Dong, Fang & Li, 2003; Oliveira et al., 2013; Xie, Schepetkin, Siemsen, Kirpotina, Wiley & Quinn, 2008), roots (Kiyohara et al., 1987; Li et al., 2012; Nergard et al., 2005; Yamada, Kiyohara, Cyong & Otsuka, 1987) and stems (Chintalwar et al., 1999). Moreover, in fruits, they have been reported for banana, lemon, pineapple, yellow passion fruit (Yapo, 2009), kiwi (Sauvageau, Hinkley, Carnachan & Sims, 2010), peach pulp (Simas-Tosin et al., 2012) and *Citrus depressa* (Tamaki, Konishi, Fukuta & Tako, 2008).

2 Materials and methods

2.1 Plant material

Ripe fruits of starfruit (*Averrhoa carambola* L.) from cultivar B10 were purchased in a local market in Curitiba, State of Paraná, Brazil.

2.2 General analytical methods

All solutions were evaporated below 60 °C under reduced pressure. Centrifugation was at 12000 x *g* for 20 min, at 10ºC. Dialyses were performed using 6-8 kDa cut-off membrane. Fruits were defatted employing chloroform-methanol (1:1) in a Soxhlet apparatus.

Fraction 50R was carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972), using $NaBH_4$ as the reducing agent, giving products with the – COOH groups of its uronic acid residues reduced to – CH_2OH.

2.3 Extraction and purification of polysaccharides

Fruits were freeze-dried, milled and defatted. Polysaccharides were extracted from the residue with boiling water, giving fraction CW (Fig. 1) as previously described by Leivas et al. (2015). Fraction CW was dissolved in distilled water and the freeze-thaw treatment was applied, giving cold-water soluble (SCW) and insoluble (PCW) fractions. In this procedure, the sample was frozen and then thawed at room temperature followed by centrifugation. This procedure was repeated until no more precipitate appeared.

Fraction SCW was dissolved in distilled water and then treated with Fehling's solutions with an equal volume of solutions A and B (solution A 250 g/L KOH, 346 g/L sodium potassium tartarate and solution B 111.5 g/L $CuSO_4.5H_2O$) (Jones & Stoodley, 1965), resulting in soluble (SFSCW) and insoluble (PFSCW) fractions, which were separated by centrifugation, neutralized with acetic acid (HOAc), dialyzed against tap water and deionized with cation exchange resin. Fraction SFSCW was further fractionated by sequential ultrafiltration (100 kDa and 50 kDa MWCO

membranes, Millipore®), yielding one homogeneous fraction (50R). Fraction 50E was later purified by ultrafiltration (10 kDa MWCO membrane, Millipore®), yielding the homogeneous fraction (10R) (Fig. 1).

Polysaccharide yields were expressed as percentages based on the weight of dried starfruit subjected to extraction (310.7 g), while the moisture and non-polar compounds were expressed as percentages based on the weight of wet starfruit (2.5 kg).

2.4 Monosaccharide analyses

Polysaccharides were hydrolyzed, reduced, acetylated and analyzed by GC-MS as previously described by Nascimento et al. (2015).

Uronic acid contents were determined using the m-hydroxybiphenyl colorimetric method (Filisetti-Cozzi & Carpita, 1991), using galacturonic acid as standard.

2.5 Determination of homogeneity and molecular weight of polysaccharides

The homogeneity and molecular weight of water-soluble polysaccharides were determined by gel permeation chromatography (GPC). Molecular weight was determined using a calibration curve, according Leivas et al. (2015).

2.6 Methylation analysis of polysaccharide

Fraction 50R was carboxy-reduced according Taylor and Conrad (1972). Carboxy-reduced sample was *O*-methylated following the Ciucanu and Kerek (1984) method. The per-*O*-methylated polysaccharide was then submitted to methanolysis in 3% HCl–MeOH (at 80 °C, 2 h) followed by hydrolysis with H_2SO_4 (0.5M, 10 h, at 100°C) and neutralization with $BaCO_3$. The resulting mixture of partially *O*-methylated products were reduced ($NaBD_4$), acetylated (acetic anhydride-pyridine) and examined by GC-MS as previously described by Nascimento et al. (2015).

2.7 Nuclear magnetic resonance (NMR) spectroscopy

^{13}C NMR and ^{13}C NMR-DEPT 135 spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer equipped with a 5 mm inverse probe. Analyses were performed at 50 °C in D_2O. Chemical shifts were expressed as δ ppm relative to CH_3 signal from acetone at δ 30.2 as internal reference.

3 Results and discussion

Ripe fruits of *A. carambola* were freeze-dried and milled. Dried pulp powder (310.7g) was defatted with chloroform-methanol (1:1), yielding a moisture and nonpolar compounds content of approximately 88% and 4%, respectively. Polysaccharides were extracted employing boiling water, giving fraction CW (7% yield).

A freeze-thaw treatment was applied in fraction CW, giving cold-water soluble (SCW, 4.3% yield) and insoluble polysaccharides (PCW, 0.6% yield). Monosaccharide analysis revealed arabinose, galactose, xylose and rhamnose as neutral sugars, and 85.2% of uronic acids in fraction SCW (Table 1), indicating the presence of pectic polysaccharides. Its ^{13}C NMR spectrum (Fig. 2A) showed signals at δ 100.1 and δ 99.3, corresponding to anomeric carbons of esterified and unesterified units of α-D-Gal*p*A, respectively. Their respective C-6 signals were seen at δ 170.7 and δ 174.8, from methyl ester carbonyl carbons and carboxyl carbons, respectively. The signal at δ 52.8 could be attributed to methyl carbons of esterified carbonyls in Gal*p*A units. The remaining α-D-Gal*p*A ring carbons were seen at δ 78.7 (*O*-substituted C-4), δ 70.5 (C-5) and δ 68.1 (C-2 and C-3, overlapped). These assignments indicated the presence of a methyl esterified homogalacturonan (HG). Moreover, it can be observed in the spectra, signals at δ 109.1, δ 107.6 and δ 107.2 corresponding to anomeric carbons of α-L-Ara*f* units and signals at δ 104.3, δ 103.4 and δ 103.2 which can be attributed to β-D-Gal*p* units, indicating the presence of a pectic arabinogalactan (AG). These signals are in agreement with published literature data (Cipriani, Mellinger, Gorin & Iacomini, 2004; Cipriani et al., 2009, Thude & Classen, 2005; Xu, Dong, Qiu, Cong & Ding, 2010).

In order to purify the arabinogalactan, fraction SCW was submitted to Fehling precipitation (Fig. 1). Homogalacturonan formed a complex with Cu^{++} and precipitated

(fraction PFSCW, 2.8% yield) while arabinogalactan does not complex with Cu^{++} and remains soluble (fraction SFSCW, 0.9% yield) in this treatment (Cantu-Jungles et al., 2014).

Monosaccharide analysis showed that fraction SFSCW contains arabinose, galactose, rhamnose and uronic acid (Table 1). Its ^{13}C NMR spectrum can be seen in Fig. 2B and showed signals at δ 109.1, δ 107.5 and δ 107.1 corresponding to anomeric carbons of α-L-Ara*f* units, and signals at δ 104.3 and δ 103.2 that can be attributed to (1→4)-linked-β-D-Gal*p* units and (1→3,6)-linked-β-D-Gal*p* units, respectively (Cipriani et al., 2006; Cipriani et al., 2009; Thude & Classen, 2005). These assignments could suggest the presence of type I (AG-I) and type II (AG-II) arabinogalactans. The presence of AG-I in starfruit had already been reported (Leivas et al., 2015). This mixture was also observed in GPC analysis, which demonstrated a heterogeneous elution profile (Fig. 3A). Thus, fraction SFSCW was further fractionated by sequential ultrafiltration through membranes with cut-offs of 100 kDa, 50 kDa and 10 kDa (Fig. 1). This strategy was highly efficient, as it produced two homogenous fractions (50R, and 10R), as could be seen by their elution profiles on GPC analysis (Fig. 3B and C). Their molecular weights were 49 kDa and 24 kDa, respectively.

In order to achieve the linkages, fraction 50R, which was obtained in higher yield, was carboxy-reduced (50R-CR). When compared with native fraction, 50R-CR had, on sugar analysis, glucose and an increased amount of galactose, indicating the presence of GlcA and GalA, respectively. Then, 50R-CR was submitted to methylation analysis (Table 2) and the main observed derivatives were those of arabinose (52.5%), which was in agreement with monosaccharide analysis (Table 1). The presence of 2,3-Me_2-Ara-ol acetate indicated (1→5)-linked Ara*f* units. The presence of 2-Me-Ara-ol acetate indicated that this arabinan was branched at *O*-3 and the derivative 2,5-Me_2-Ara-ol acetate indicated the presence of (1→3)-linked Ara*f* units, probably present as side chains. The nonreducing terminals consisted of Ara*f* and a small amount of Ara*p*. The presence of Ara in its pyranosidic form seems to be a typical feature of type II arabinogalactans (Capek, 2008; Goellner, Utermoehlen, Kramer & Classen, 2011; Odonmažig, Ebringerová, Machová & Alföldi, 1994; Oliveira et al., 2013; Ponder & Richards, 1997; Willför, Sjöholm, Laine & Holmbom, 2002). High amounts of arabinose units were also observed in AG-II of *Stevia*

rebaudiana (Oliveira et al., 2013), *Diospyros kaki* (Duan et al., 2003) and *Nerium indicum* (Dong & Fang, 2001) leaves, of Herba Asari roots (Li et al., 2012) and of *Viscum album* 'berries' (Wagner & Jordan, 1988).

Moreover, galactose was present only in the pyranosidic form and terminal, (1→3)-, (1→6)- and (1→3,6)-linked units. Lower content of 3-*O*-substituted than that of 3,6-di-*O*-substituted Gal*p* units is a usual finding for type II arabinogalactans (Capek, 2008; Cipriani et al., 2006; Dong & Fang, 2001; Duan et al., 2003; Goellner et al., 2011; Odonmažig et al., 1994; Ponder & Richards, 1997; Simas-Tosin et al., 2012; Wang, Zheng, Zuo & Fang, 2005; Willför et al., 2002). However, starfruit's AG-II showed high amounts of (1→6)-linked Gal*p* units. This feature was also observed for some pectic AG-II from other sources (Capek, 2008; Dong & Fang, 2001; Kiyohara et al., 1987; Oliveira et al., 2013; Raju & Davidson, 1994; Wagner & Jordan, 1988), where a (1→6)-β-galactopyranose backbone was proposed.

Furthermore, the derivatives 3,4-Me_2- and 3-Me-Rha-ol acetates demonstrated the presence of (1→2)- and (1→2,4)-linked Rha*p* units. This latter could be the insertion point of AG-II in the rhamnogalacturonan backbone. The 2,3,6-Me_3-Gal-ol acetate indicated that (1→4)-linked Gal*p*A units were also present. The finding of (1→4)-linked Gal*p*A together with (1→2)- and (1→2,4)-linked Rha*p* confirms the presence of type I rhamnogalacturonan. The methylation analysis also had 2,3,4,6-Me_4-Glc-ol acetate, which arose from terminal Glc*p*A units. The presence of Glc*p*A linked to *O*-6 or *O*-3 of Gal*p* residues in arabinogalactan moiety has been already described (Cipriani et al., 2006; Goellner et al., 2011; Urbas, Bishop & Adams, 1963; Willför et al., 2002).

^{13}C NMR spectra of 50R (Fig. 4A) and 10R (Fig. 4B) showed qualitatively the presence of the same peaks. Anomeric signals of AG-II could be seen at δ 109.1, δ 107.5 and δ 107.1, attributed to C-1 of terminal, 5- and 3,5-linked α-L-Ara*f* units, respectively, at δ 103.4 corresponding to C-1 of 3- and 3,6-linked β-D-Gal*p* units, while that at δ 103.2 is from 6-linked β-D-Gal*p* units. Moreover, the signals at δ 96.8/97.7 could be attributed to anomeric carbons of terminal α-Glc*p*A units (Odonmažig, Badgaa, Ebringerová, Mihálov & Alföldi, 1990; Wang et al., 2005). 13C NMR- DEPT 135 spectrum of 50R (Fig. 4A) showed downfield shifted inverted signals at δ 69.2, corresponding to substituted C-6 of the β-D-Galp and at δ 66.6 and δ 66.2 corresponding to substituted C-5 of α-L-Araf units. Moreover, inverted signals

at δ 61.2, δ 62.5 and δ 63.3 correspond to non-substituted C-6 of the β-D-Gal*p* units, non-substituted C-5 of α-L-Ara*f* units and non-substituted C-5 of Ara*p*, respectively (Delgobo, Gorin, Tischer & Iacomini, 1999; Gorin & Mazurek, 1975; Thude & Classen, 2005). Signals from type I rhamnogalacturonan core could be seen at δ 99.4 and δ 98.4, attributed to C-1 of α-L-Rha*p* and α-D-Gal*p*A units, respectively, and a signal at δ 16.6 corresponding to C-6 of α-L-Rha*p* (Wang, Dong, Zuo & Fang, 2003).

Therefore, the above results suggested that fraction 50R and 10R contains type II arabinogalactans. The presence of rhamnose and galacturonic acid suggested that these type II arabinogalactans were anchored in type I rhamnogalacturonans. Type II arabinogalactans linked to type I rhamnogalacturonans have also been found in *Viscum album* (Wagner & Jordan, 1988), *M. ilicifolia* (Cipriani et al., 2006), and *S. rebaudiana* (Oliveira et al., 2013). Concerning fruits, to date pectic type II arabinogalactans have only been recently isolated and chemically characterized from peach pulp by Simas-Tosin et al. (2012). Their presence has been cited in other fruits, such as banana, kiwi, lemon, pineapple, yellow passion fruit (Sauvageau et al., 2010; Yapo, 2009), and *Citrus depressa* (Tamaki et al., 2008). Regarding biological activity, some AG-II presented protective effects against gastric lesions induced by ethanol in mice (Cipriani et al., 2006), *in vitro* antiviral activity against Herpes simplex virus type-1 (HSV-1) (Oliveira et al., 2013), and activation of peritoneal macrophages in mice (Simas-Tosin et al., 2012).

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TABLE 1 – MONOSACCHARIDE COMPOSITION OF FRACTIONS OBTAINED FROM THE FRUIT OF STARFRUIT (*Averrhoa carambola* L.)

Fractions	**Yield**[b]	**Monosaccharide composition (%)**[a]					
		Rha	Ara	Xyl	Gal	Glc	Uronic acid[c]
SCW	4.3	1.6	9.0	1.8	2.4	-	85.2
SFSCW	0.9	1.4	49.8	-	10.9	-	37.8
50R	0.03	4.3	56.2	-	37.4	-	2.0
50R-CR[d]	-	4.4	52.3	-	40.4	2.9	-
10R	0.01	2.8	65.8	-	28.5	-	3.0

[a] % of peak area relative to total peak areas, determined by GC–MS.

[b] Determined spectrofotometrically using the m-hydroxybiphenyl method (Filisetti-Cozzi & Carpita, 1991).

[c] Not determined.

[d] Fraction 50R was carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972).

TABLE 2 – LINKAGE TYPES BASED ON ANALYSIS OF PARTIALLY O-METHYL ALDITOL ACETATES OF FRACTION 50R

Partially *O*-methylalditol acetate	Linkage type[b]	50R	
		mol%[c]	%
Arabinose			
2,3,5-Me_3-Ara[a]	Ara*f*-(1→	15.9	30.3
2,3,4-Me_3-Ara	Ara*p*-(1→	2.4	4.6
2,5-Me_2-Ara	→3)-Ara*f*-(1→	8.0	15.2
2,3-Me_2-Ara	→5)-Ara*f*-(1→	14.0	26.7
2-Me-Ara	→3,5)-Ara*f*-(1→	12.2	23.2
		52.5	100.0
Galactose			
2,3,4,6-Me_4-Gal	Gal*p*-(1→	5.0	12.3
2,4,6-Me_3-Gal	→3)-Gal*p*-(1→	3.5	8.6
2,3,6-Me_3-Gal	→4)-Gal*p*-(1→	1.2	2.9
2,3,4-Me_3-Gal	→6)-Gal*p*-(1→	20.4	50.1
2,4-Me_2-Gal	→3,6)-Gal*p*-(1→	10.6	26.0
		40.7	100.0
Rhamnose			
3,4-Me_2-Rha	→2)-Rha*p*-(1→	2.5	58.1
3-Me-Rha	→2,4)-Rha*p*-(1→	1.8	41.9
		4.3	100.0
Glucose			
2,3,4,6-Me_4-Glc	Glc*p*-(1→	2.5	100.0

[a] 2,3,5-Me_3-Ara = 2,3,5-tri-*O*-Methylarabinitolacetate, etc.

[b] Based on derived *O*-methylalditol acetates.

[c] According Pettolino et al. (2012). Samples were carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972), prior to methylation analysis.

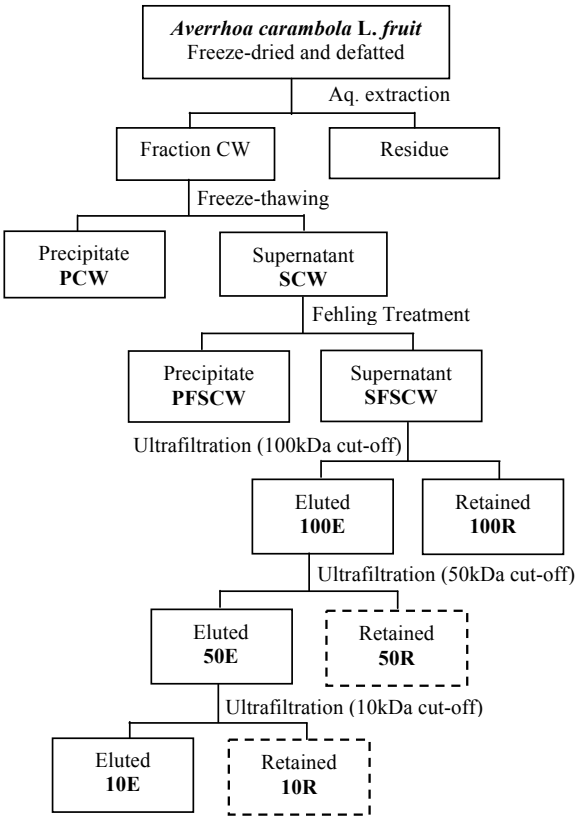

FIGURE 1 – SCHEME OF EXTRACTION AND FRACTIONATION OF WATER SOLUBLE POLYSACCHARIDES FROM STARFRUIT (*Averrhoa carambola* L.)

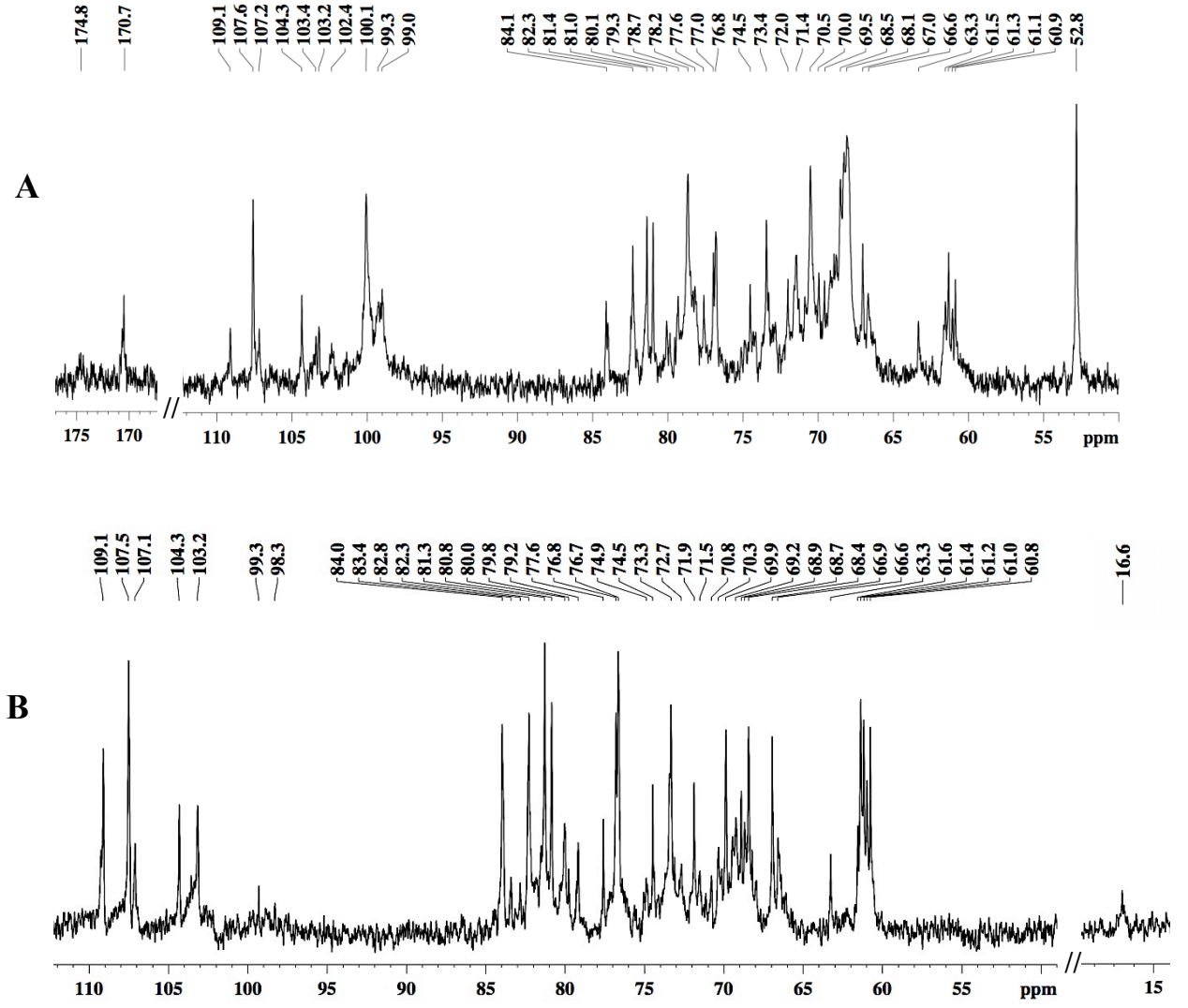

FIGURE 2 – ^{13}C-NMR SPECTRA OF FRACTIONS SCW (A) AND SFSCW (B), IN D_2O AT 50 ºC

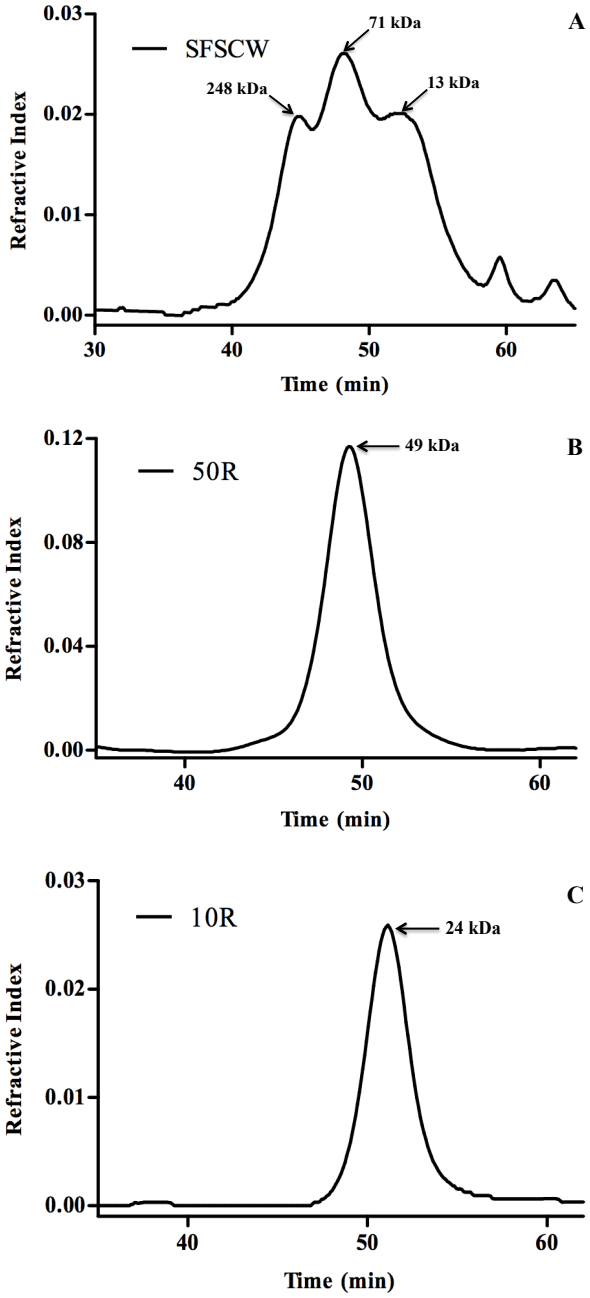

FIGURE 3 – GPC ELUTION PROFILES OF FRACTIONS SFSCW (A), 50R (B) AND 10R (C). REFRACTIVE INDEX DETECTOR

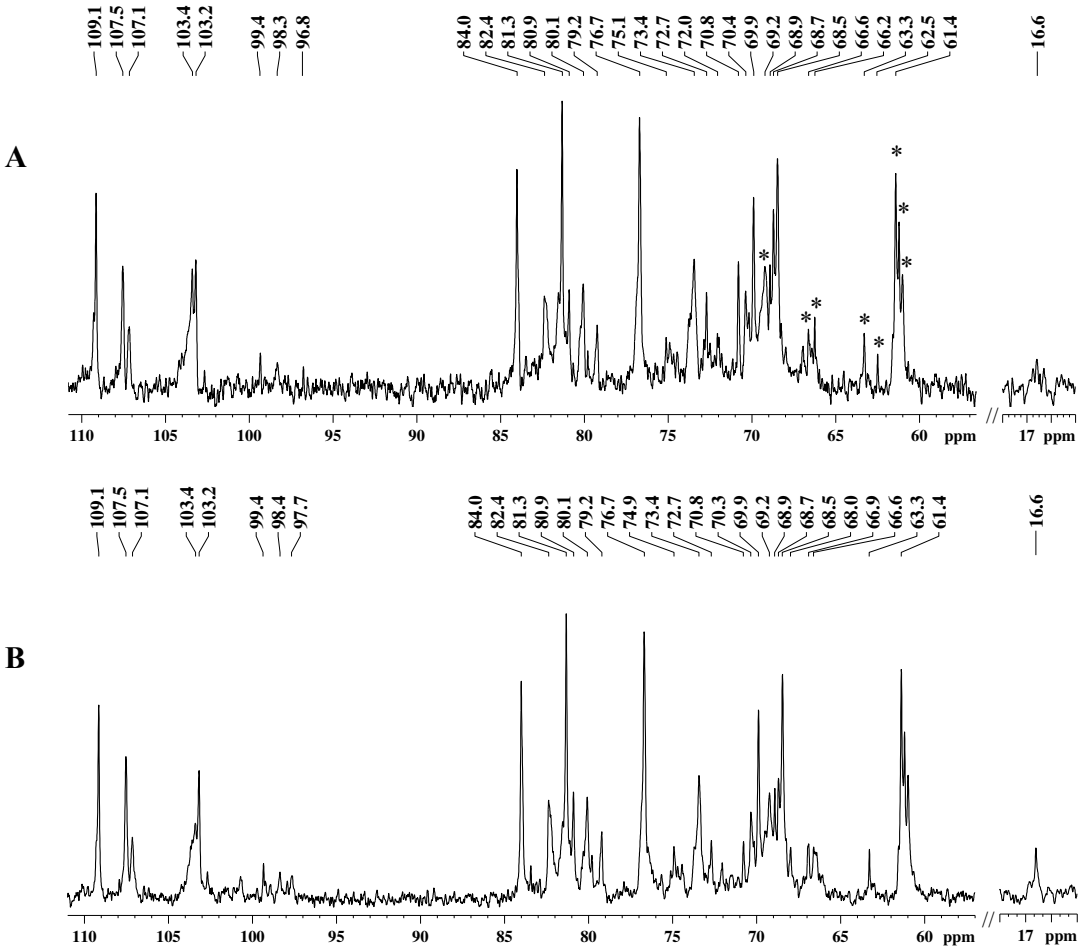

FIGURE 4 – ^{13}C-NMR SPECTRA OF FRACTIONS 50R (A) AND 10R (B), IN D_2O AT 50 ºC. INVERTED SIGNALS IN DEPT-135 EXPERIMENT ARE MARKED WITH ASTERISK

ARTIGO IV

Structural characterization of a fucogalactoxyloglucan from edible starfruit (*Averrhoa carambola* L.)

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ABSTRACT

Polysaccharides from ripe fruits of starfruit (*Averroa carambola* L.) were obtained following alkaline extraction. These were analyzed by sugar composition, gel permeation chromatography, methylation and 2D NMR spectroscopy analyses. Results showed the presence of a xyloglucan and a heteroxylan. The xyloglucan was separated by freeze-thawing, Fehling treatment, anion exchange chromatography and was characterized as a fucogalactoxyloglucan, with an M_w of 69kDa.

Keywords: Starfruit, *Averrhoa carambola*, NMR, Fucogalactoxyloglucan, Heteroxylan.

1. Introduction

In land plants, the primary cell walls are composed predominantly of polysaccharides (cellulose, hemicelluloses and pectic substances) (Hsieh & Harris, 2012; Yapo & Koffi, 2008). Among hemicellulosic polysaccharides, xyloglucans represent an important component (Hayashi, 1989) and have a cellulosic (1→4)-linked β-D-glucan backbone partially substituted at *O*-6 by α-D-xylosyl residues (Carpita & Gibeault, 1993; Hayashi, 1989; McNeil, Darvill, Fry & Albersheim, 1984; Renard, Lemeunir & Thibault, 1995). Depending on the source, xyloglucans can have

differences in their side chains (McNeil et al., 1984). For example, in fucogalactoxyloglucans, some of the xylosyl side chains can be substituted at *O*-2 by terminal β-D-Gal*p*-(1→ and/or α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→ (Hayashi, 1989; Hsieh & Harris, 2009; McNeil et al., 1984; Renard et al., 1995). Complex side chains were observed in xyloglucans of bilberry, composed by β-D-Xyl*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* (Hilz et al., 2007). Moreover, members of the Solanaceae family produce xyloglucans that contain α-L-Ara*f* and/or β-D-Gal*p* residues but lack α-L-Fuc*p* residues (Hayashi, 1989; Hoffman et al., 2005; Jia, Qin, Darvill & York, 2003; Ring & Selvendran, 1981). In dicotyledonous fruits, fucogalactoxyloglucans have already been described for apple, avocado, fig, grape, kiwi, mandarin, peach, persimmon, plum, prune and strawberry (Cutillas-Iturralde et al., 1998; Doco, Williams, Pauly, O'Neill & Pellerin, 2003; Ito & Kato, 2002; Kato, Uchida, Ito & Mitsuishi, 2001; Ray, Vigouroux, Quémener, Bonnin & Lahaye, 2014; Renard & Ginies, 2009; Renard, Lomax & Boon, 1992; Watt, Brasch, Larsen & Melton 1999). Fucogalactoxyloglucans have also been reported for banana and pineapple, despite being monocotyledonous fruits (Ito & Kato, 2002; Kato et al., 2001). However, there are no studies dealing with starfruit's xyloglucans.

Starfruit (*Averrhoa carambola* L.) belongs to Oxalidaceae family, is a dicotyledon that occurs in Malaysia, Asian countries and in tropical areas of America (Dasgupta, Chakraborty & Bala, 2013; Manda, Vyas, Panday & Singha, 2012; O'Hare, 1993; Soncini et al., 2011). In Brazil this edible fruit is known as carambola and is consumed as fresh fruit or as juice fruit (Ferreira, Fernandes, Galende, Cortez & Bazotte, 2008). Regarding to starfruit's polysaccharides, Leivas, Iacomini and Cordeiro (2015) described a pectic polysaccharide formed by a type I rhamnogalacturonan containing arabinan and type I arabinogalactan as neutral side chains, a pectic type II arabinogalactan (Leivas, Iacomini & Cordeiro, 2016a) and a substituted galacturonan (Leivas et al., 2016b). Thus, this paper reports the isolation, composition and structural features of a fucogalactoxyloglucan from *A. carambola* fruits.

2. Materials and methods

2.1. Plant material

Ripe fruits of starfruit (*Averrhoa carambola* L.) from cultivar B10 were purchased in the local market of Curitiba, State of Paraná, Brazil.

2.2. General analytical methods

All solutions were evaporated below 60 °C under reduced pressure. Dialyses were performed using 12-14 kDa cut-off membrane.

The total lipid quantification was performed by extraction employing chloroform-methanol (1:1) as solvent through Soxhlet apparatus.

The fraction was carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972), using $NaBH_4$ as the reducing agent, giving products with the – COOH groups of its uronic acid residues reduced to – CH_2OH.

2.3. Extraction and purification of cell wall polysaccharides

Fruits were washed, cut and the seeds were manually removed. The fruits were freeze-dried and milled. Dried pulp powder (310.7 g) was defatted with chloroform-methanol (1:1). Polysaccharides from starfruit were extracted with boiling water and aq. 10% NaOH (Fig. 1). The alkaline extract (CK) was purified, giving SFSCK (1.9% yield) and PFSCK (0.5% yield) fractions, as previously described by Leivas et al. (2015).

Fraction PFSCK was further purified by anion exchange chromatography. It was dissolved in distilled water (50 mg/mL), centrifuged (12000 x *g*, 10 min at 10 ºC) and the supernatant applied to a DEAE-Sepharose Fast Flow column (3.0 cm×25 cm). The column was eluted with distilled water (giving fraction XLG), followed by 4.0 M NaCl solution (giving fraction PFSCK-4M) (Fig. 1) at a flow rate of 1.5 mL/min. Polysaccharides in the eluted fractions were detected using phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Fraction XLG was concentrated and freeze-dried.

The yields of polysaccharides were expressed as percent based on weight of dried starfruit that was submitted to extraction (310.7 g), while the moisture and non-

polar compounds were expressed as percent based on the weight of wet starfruit (2.5 kg).

2.4. Sugar composition

Neutral sugars were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 8 h at 100 °C, reduced with $NaBH_4$ (Wolfrom &Thompson, 1963a) and acetylated with acetic anhydride-pyridine (1:1, v/v) for 18 h at 25 °C (Wolfrom & Thompson, 1963b). The resulting alditol acetates were analyzed by GC-MS following the conditions employed by Leivas et al. (2015).

Uronic acids were determined using the *m*-hydroxybiphenyl colorimetric method (Filisetti-Cozzi & Carpita, 1991) and concentrations were estimated from a calibration curve using galacturonic acid as standard.

2.5. Determination of homogeneity and molecular weight of polysaccharides

The homogeneity and molecular weight of water-soluble polysaccharides were determined by gel permeation chromatography (GPC). The procedure was carried out as previously reported by Leivas et al. (2015).

2.6. Methylation analysis of polysaccharide

Fraction PFSCK was carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972) and methylated according to the Ciucanu and Kerek (1984) method. The per-*O*-methylated polysaccharide was then submitted to methanolysis in 3% HCl–MeOH (at 80 °C, 2 h) followed by hydrolysis with H_2SO_4 (0.5M, 12 h, at 100°C) and neutralization with $BaCO_3$. The resulting mixture of partially *O*-methylated products were reduced ($NaBD_4$) and acetylated (acetic anhydride-pyridine). The products (partially *O*-methylated alditol acetates) were examined by GC-MS using a DB-225 capillary column (30 m x 0.25 mm i.d.), held at 50 ºC during injection and then programmed to increase to 210 ºC, at a rate of 40 ºC/min. The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (Sassaki, Gorin, Souza, Czelusniak & Iacomini, 2005).

2.7. Nuclear magnetic resonance (NMR) spectroscopy

2D NMR experiments (coupled, decoupled and edited HSQC and TOCSY) were acquired at 50 °C on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^{1}H at 400.13 MHz and ^{13}C at 100.61 MHz, equipped with a 5-mm multinuclear inverse detection probe with z-gradient. Samples (20 mg) were deuterium-exchanged three times by freeze-drying with D_2O solutions, finally dissolved in D_2O and transferred into 5-mm NMR tube. Chemical shifts were expressed as δ ppm relative to CH_3 signal from acetone at δ 30.2 and 2.224 for ^{13}C and ^{1}H signals, respectively, as internal reference.

3. Results and discussion

Ripe fruits of *A. carambola* were freeze-dried and milled yielding a moisture of approximately 88%. Dried pulp powder (310.7g) was defatted with chloroform-methanol (1:1), yielding nonpolar compounds content of approximately 4%.

Dried and defatted *A. carambola* fruits were submitted to sequential aqueous and alkaline extractions, giving rise to fractions CW (7% yield) and CK (5% yield) respectively. A fraction named PFSCK (0.5% yield) was obtained from fractionation of CK, as previously described by Leivas et al. (2015) and depicted in Fig. 1. Its monosaccharide analysis indicated Xyl:Glc:Ara:Gal:Fuc as neutral sugars in 37.8:33.3:10.2:9.6:2.3 molar ratios and 6.8% of uronic acids. Fraction PFSCK was carboxy-reduced and submitted to monosaccharide analysis by GC-MS. This procedure converted the uronic acid in their corresponding neutral sugar. Carboxy-reduced fraction demonstrated an increase in the percentage of glucose in the GC-MS analysis, indicating that the uronic acid present was the glucuronic acid.

Methylation analysis of PFSCK (Table 1) was performed to determine the position of its glycosidic linkages. The main observed derivatives were those of xylose and glucose, which were in agreement with monosaccharide composition. For glucose derivatives, 2,3,6-Me_3- and 2,3-Me_2-Glc-ol acetates were observed, indicating a (1→4)-linked glucan carrying branches exclusively at *O*-6. Derivatives 2,3,4-Me_3- and 3,4-Me_2-Xyl-ol acetates arose from terminal and (1→2)-linked Xyl*p* units, respectively. Methylated derivatives of galactose were 2,3,4,6-Me_4- and 3,4,6-

Me_3-Gal-ol acetates, indicating terminal and (1→2)-linked Gal*p* units, respectively. Terminal Fuc*p* units were also observed. These derivatives were observed for xyloglucans by Arruda et al. (2015), Busato et al. (2005), Lucyszyn et al. (2011) and Ray et al. (2004). Moreover, other methylated derivatives of xylose were also present, that is 2,3-Me_2- and 3-Me-Xyl-ol acetates, which arose from 4-*O*- and 2,4-di-*O*-substituted Xyl*p* units, respectively. The presence of 2,3-Me_2-Ara-ol acetate indicated (1→5)-linked Ara*f* units. Furthermore, the methylation analysis also had 2,3,4-Me_3-Ara-ol acetate and 2,3,4,6-Me_4-Glc-ol acetate, which arose from terminal Ara*p* and probably from terminal Glc*p*A units, respectively. The presence of (1→4)-linked-Xyl*p* substituted at *O*-2 have previously been observed for heteroxylans extracted from leaves of *M. ilicifolia* (Cipriani et al., 2008) and pulp of tamarillo fruits (Nascimento et al., 2013). Thus, these data could suggest the presence of a xyloglucan together with a heteroxylan. This mixture was observed in GPC analysis (Fig. 2), which demonstrated a heterogeneous elution profile.

Fraction PFSCK was also examined by 2D NMR spectroscopy. The $^{1}H/^{13}C$ HSQC (Fig. 3A) spectrum contained nine distinct signals in the anomeric region, while their anomericity was seen by the determination of $J_{C\text{-}1,H\text{-}1}$ coupling constants (Fig. 3B, Table 2). Signals at δ 102.3/4.53, 104.4/4.57, 103.2/4.62, 98.7/4.94, 98.6/5.14 and 99.4/5.28 could be attributed to C1/H1 of β-D-Glc*p*, terminal β-D-Gal*p*, substituted β-D-Gal*p*, terminal α-D-Xyl*p*, substituted α-D-Xyl*p* and α-L-Fuc*p*, which belong to xyloglucan. All the signals were compared to literature values for similar polysaccharides (Arruda et al., 2015; Bubb, 2003; Busato et al., 2005; Duus, Gotfredsen & Bock, 2000; Hilz et al., 2007; Hoffman et al., 2005; Lucyszyn et al., 2011; Sassaki et al., 2014). Furthermore, it can also be seen in Fig. 3A signals at δ 101.6/4.49, indicating C1/H1 of β-D-Xyl*p* units. This H1 signal coupled to other hydrogens at 4.13, 3.79, 3.56 and 3.40 in TOCSY correlation map (Fig 3C). These hydrogens, in turn coupled with their carbons at 62.9, 76.3, 73.7 and 72.9 in HSQC spectrum (Fig 3A). A comparison with literature (Cipriani et al., 2008; Cordeiro, Almeida & Iacomini, 2015; Simas-Tosin et al., 2013) confirms they arose from C5/H5, C4/H4, C3/H3 and C2/H2 of (1→4)-linked β-D-Xyl*p* units present in the heteroxylan. Moreover, signals at δ 100.4/4.71 and 96.6/5.39 could be attributed to β-L-Ara*p* units and α-D-Glc*p*A units (Delgobo, Gorin, Tischer & Iacomini, 1999).

In order to purify the xyloglucan, fraction PFSCK was submitted to anion exchange chromatography (Fig. 1). This strategy was highly efficient for purification of the xyloglucan, giving fraction XLG (69 kDa molecular weight), as could be seen by its monosaccharide composition and 2D NMR spectroscopy (Fig. 4). Monosaccharide composition showed Glc:Xyl:Gal:Fuc as neutral sugars in 50.8:29.0:12.2:8.0 molar ratio, comparable with those observed for banana, strawberry (Ito & Kato, 2002), garlic, onion (Ohsumi & Hayashi, 1994) and apple pomace (Watt et al. 1999), where glucose was the major neutral monosaccharide, followed by xylose, galactose and fucose. The presence of galactose and fucose indicated a fucogalactoxyloglucan.

The $^{1}H/^{13}C$ HSQC spectrum of fraction XLG (Fig. 4) showed six distinct signals in the anomeric region, and it is possible to observe the disappearance of the C1 signals at δ 101.6/4.49 that belonged to the heteroxylan. Signals at δ 102.3/4.55, 104.4/4.56, 103.1/4.62, 98.8/4.94, 98.6/5.15 and 99.3/5.27 corresponded to C1/H1 of β-D-Glc*p,* terminal β-D-Gal*p,* 2-*O*-substituted β-D-Gal*p,* terminal α-D-Xyl*p,* substituted α-D-Xyl*p* and α-L-Fuc*p* units, respectively (Busato et al., 2005; Hoffman et al., 2005; Watt et al., 1999). Edited HSQC experiment (Fig 4) gave negative signals at δ 66.3/3.99 and 66.5/3.91, attributed to C6/H6 of 6-*O*-substituted β-D-Glc*p* units. Signals at δ 61.0/3.78 and 61.5/3.57 could be assigned to C6/H6 of β-D-Gal*p* and C5/H5 of α-D-Xyl*p* units, respectively (Jia et al., 2003; Lucyszyn et al., 2011; York, Harvey, Guillen, Albersheim & Darvill, 1993). Signals at δ 15.9/1.26 (Fig. 4 insert) corresponded to C6/H6 of α-L-Fuc*p* (Busato et al., 2005; Watt et al., 1999). These data are consistent with those found for fucogalactoxyloglucans from other plant sources (Busato et al., 2005; Hoffman et al., 2005; Wagner, Stuppner, Schafer & Zenk, 1988; Watt et al., 1999).

On the whole, the xyloglucan isolated from starfruit is a fucogalactoxyloglucan, which is the most common type of xyloglucan in the primary cell walls of dicotyledons. In fruits, fucogalactoxyloglucan has already been reported for dicotyledonous apple, avocado, fig, grape, kiwi, mandarin, peach, persimmon, plum, prune and strawberry (Cutillas-Iturralde et al., 1998; Doco et al., 2003; Ito & Kato, 2002; Kato et al., 2001; Ray et al., 2014; Renard & Ginies, 2009; Renard et al., 1992; Watt et al., 1999) and in monocotyledonous banana and pineapple (Ito & Kato, 2002).

4. Conclusions

The presence of a fucogalactoxyloglucan and a heteroxylan was reported herein for *A. carambola* fruits. The fucogalactoxyloglucan was purified by freeze-thawing, Fehling treatment and anion exchange chromatography and chemically characterized by monosaccharide and 2D NMR analyses. The obtained data showed that its structural features were similar to those found in other xyloglucans present in fruits.

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TABLE 1 – LINKAGE TYPES BASED ON ANALYSIS OF PARTIALLY *O*-METHYL ALDITOL ACETATES OF FRACTION PFSCK

Partially *O*-methylalditol acetate	**Linkage type[b]**	**mol%[c]**
2,3,4-Me_3-Ara[a]	Ara*p*-(1→	9.9
2,3-Me_2-Ara	→5)-Ara*f*-(1→	1.3
2,3,4-Me_3-Fuc / 2,3,4-Me_3-Xyl	Fuc*p*-(1→ / Xyl*p*-(1→	6.4
2,3- / 3,4-Me_2-Xyl	→4)-Xyl*p*-(1→ / →2)-Xyl*p*-(1→	23.6[d]
3-Me-Xyl	→2,4)-Xyl*p*-(1→	9.9
2,3,4,6-Me_4-Gal	Gal*p*-(1→	6.8
3,4,6-Me_3-Gal	→2)-Gal*p*-(1→	5.1
2,3,4,6-Me_4-Glc	Glc*p*-(1→	1.4[e]
2,3,6-Me_3-Glc	→4)-Glc*p*-(1→	15.7
2,3-Me_2-Glc	→4,6)-Glc*p*-(1→	19.9

[a] 2,3,4-Me_3-Ara = 2,3,4-tri-*O*-Methylarabinitolacetate, etc.

[b] Based on derived *O*-methylalditol acetates.

[c] Quantified according to their effective carbon response (Sweet et al., 1975). Samples was carboxy-reduced by the carbodiimide method (Taylor and Conrad, 1972), prior to methylation analysis.

[d] The ratio of 2,3-/3,4-Me_2-Xyl was estimated by their fragmentation patterns in GC-MS and was 1.0:0.3.

[e] Arises from Glc*p*A.

TABLE 2 – ANOMERIC ^{1}H AND ^{13}C CHEMICAL SHIFTS AND $J_{C-1,H-1}$ COUPLING CONSTANTS OF THE MONOSACCHARIDE UNITS FOUND IN FRACTION PFSCK

Residues	**Chemical shifts (ppm)[a]**		**$J_{C-1,H-1}$ (Hz)[b]**
	C-1	**H-1**	
β-D-Gal*p*	104.4	4.57	160
β-D-Gal*p* substituted	103.2	4.62	161
β-D-Glc*p*	102.3	4.53	162
β-D-Xyl*p*	101.6	4.49	161
β-L-Ara*p*	100.4	4.71	162
α-L-Fuc*p*	99.4	5.28	172
α-D-Xyl*p*	98.7	4.94	171
α-D-Xyl*p* substituted	98.6	5.14	174
α-D-Glc*p*A	96.6	5.39	178

[a] $^{1}H/^{13}C$ chemical shifts observed in decoupled HSQC spectrum.

[b] Determined by $^{1}H/^{13}C$ coupled HSQC spectrum.

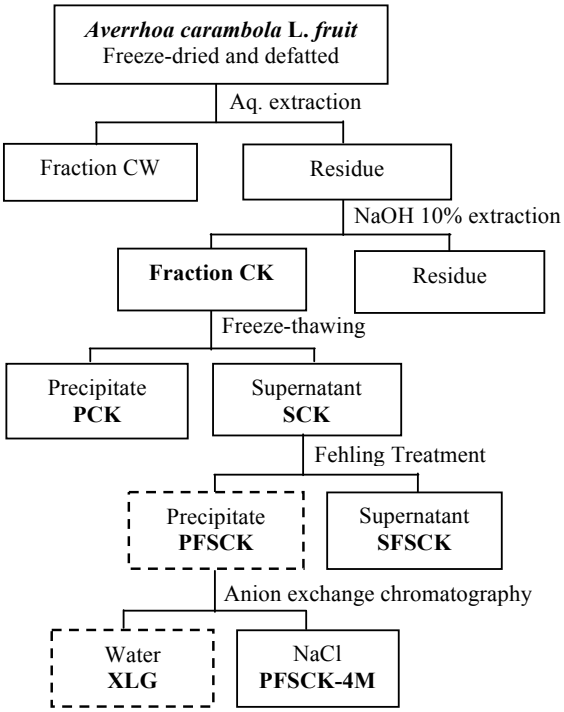

FIGURE 1 – SCHEME OF EXTRACTION AND FRACTIONATION OF THE FUCOGALACTOXYLOGLUCAN FROM STARFRUIT (*Averrhoa carambola* L.)

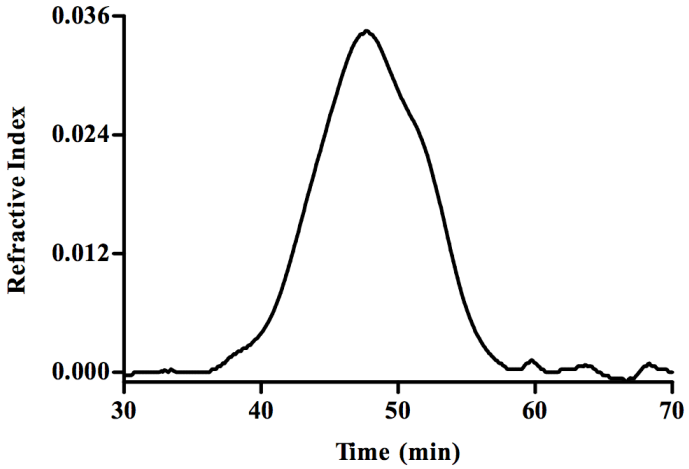

FIGURE 2 – GPC ELUTION PROFILE OF FRACTION PFSCK. REFRACTIVE INDEX DETECTOR

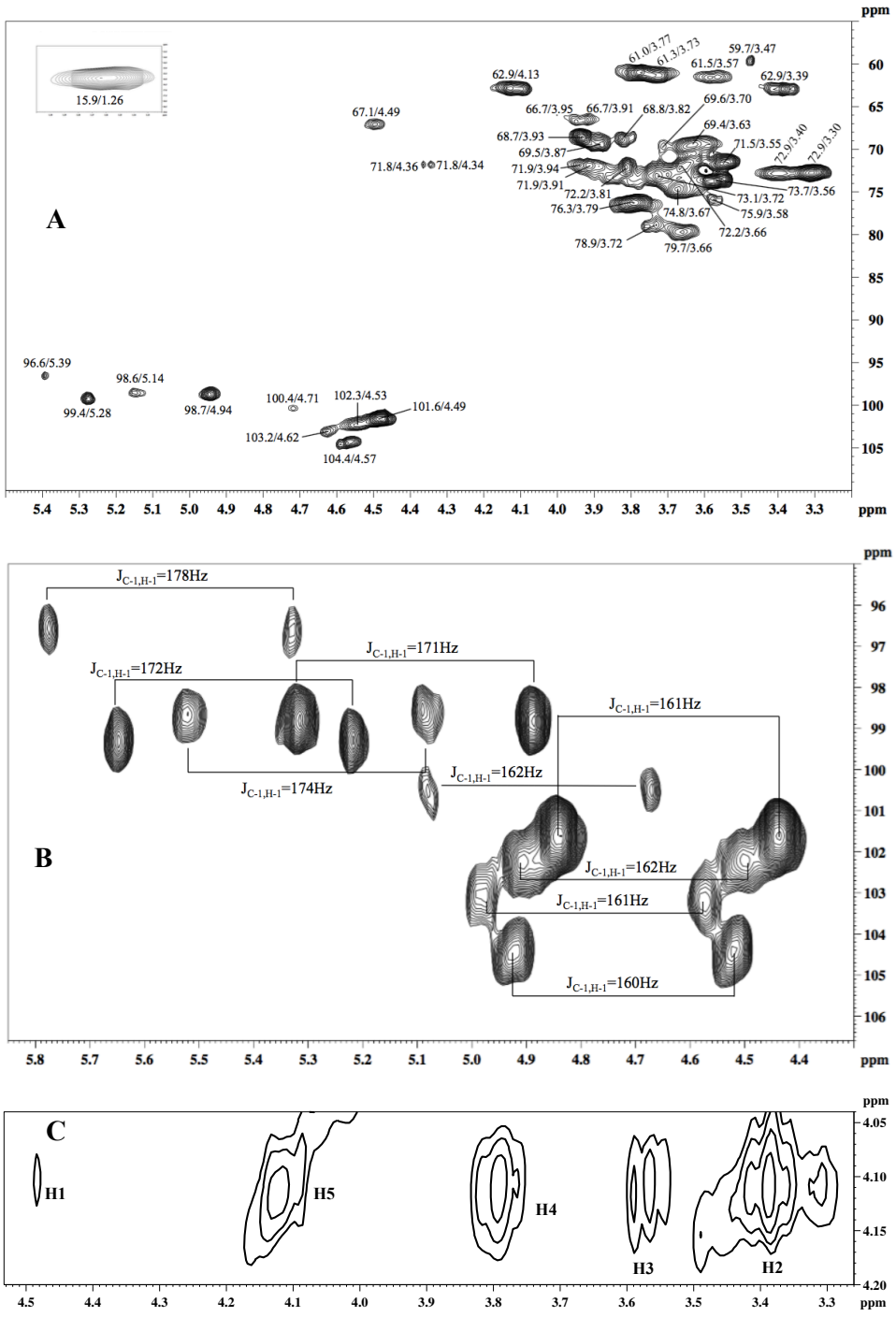

FIGURE 3 – NMR ANALYSES OF FRACTION PFSCK, IN D_2O AT 50 °C. (A) 2D $^{1}H/^{13}C$ HSQC SPECTRUM; (B) ANOMERIC $^{1}H/^{13}C$ COUPLED HSQC SPECTRUM; (C) ^{1}H-^{1}H TOCSY SPECTRUM SHOWING THE COUPLED B-D-XYL*P* HYDROGENS. THE INSERT IN A REPRESENTS C-6 REGION OF THE FUC*P* UNITS

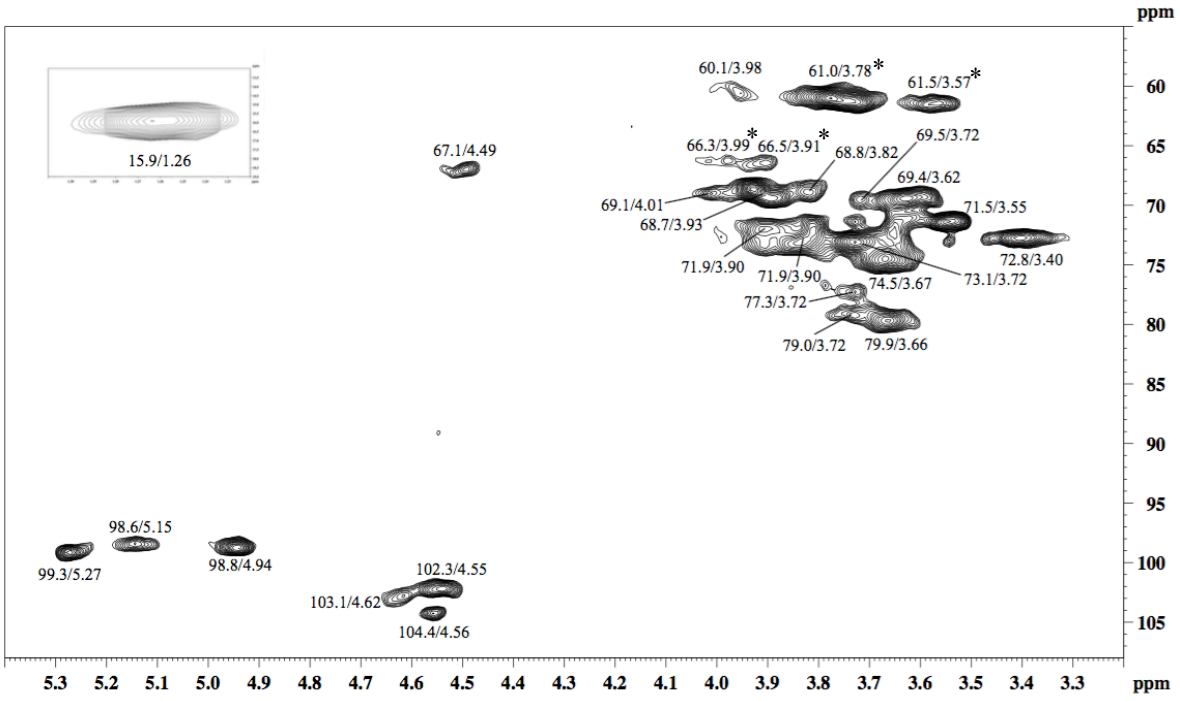

FIGURE 4 – 2D $^{1}H/^{13}C$ HSQC SPECTRUM OF FRACTION XLG, IN D_2O AT 50 °C. THE INSERT REPRESENTS THE C-6 REGION OF THE FUC*P* UNITS. INVERTED SIGNALS IN EDITED HSQC ARE MARKET WITH ASTERISK

5 POLISSACARÍDEOS EXTRAÍDOS DA POLPA DA GRAVIOLA

A seguir serão descritos as etapas de extração, purificação e caracterização dos polissacarídeos obtidos da polpa da graviola (*Annona muricata* L.), cujos resultados ainda não foram publicados.

5.1 MATERIAL DE ESTUDO

A graviola (*Annona muricata* L.) da cultivar Morada, foi cedida por produtores do município de Mirandópolis/SP. A graviola (3,7 kg) foi descascada e cortada, sendo desprezadas a casca e as sementes. A polpa foi congelada e liofilizada rendendo 619 g de polpa seca, apresentando aproximadamente 83% de umidade.

5.2 EXTRAÇÃO LIPÍDICA

O material seco foi deslipidificado e despigmentado em Soxhlet, com clorofórmio-metanol (1:1, v/v) sob refluxo, a 60°C, durante 5 dias, resultando em 498,6 g de material residual e 24,3% de compostos apolares.

5.3 EXTRAÇÃO DOS POLISSACARÍDEOS

5.3.1 Extração aquosa a quente

O resíduo deslipidificado foi submetido a repetidas extrações aquosas sequenciais (8 vezes), com agitação mecânica, por 2 horas em banho com água fervente. O material obtido de cada extração foi filtrado, gerando um sobrenadante e um resíduo. O sobrenadante foi concentrado sob pressão reduzida até pequeno volume e precipitados com etanol (3 x vol.). O precipitado foi separado do

sobrenadante por centrifugação (10.000 rpm / 20 min / 10 °C), dialisados em água corrente (6-8 kDa / 24 horas) e liofilizado.

5.3.2 Extração alcalina

O resíduo das extrações aquosas foi submetido a repetidas extrações com NaOH (10%), com agitação mecânica em banho com água fervente, por 2 horas. Os extratos foram reunidos, filtrados, neutralizados com ácido acético glacial, dialisados em água corrente (12-14 kDa / 48 horas) e liofilizados.

5.4 PURIFICAÇÃO DOS POLISSACARÍDEOS

Os polissacarídeos obtidos, a partir das extrações aquosas e alcalinas, foram submetidos a processos sequenciais de purificação, através de processos como congelamento e degelo (GORIN e IACOMINI, 1984), precipitação por formação de complexo solúvel e insolúvel em Cu^{2+} (precipitação com solução de Fehling) (JONES e STOODLEY, 1965), ultrafiltração e diálise em membranas e tratamento com enzimas (FIGURAS 10 e 11). Os processos de purificação foram monitorados através do perfil cromatográfico obtido através de cromatógrafo de exclusão estérica de alta *performance* (HPSEC) e ressonância magnética nuclear.

5.4.1 Tratamento com α-amilase

As frações polissacarídicas obtidas das extrações aquosas (GW) (FIGURA 10) e as frações obtidas após o fracionamento por congelamento e degelo das extrações alcalinas (SGK e PGK) (FIGURA 11), foram tratadas com α–amilase de *Bacillus licheniformis* (SIGMA®) para remoção dos teores de amido. A reação ocorreu com 100 unidades/mL de enzima em água, a 37 °C, por 24 h. O acompanhamento da degradação do amido foi realizado pelo teste do lugol. Após a reação, as frações foram dialisadas (6-8 kDa / 24 horas) e liofilizadas.

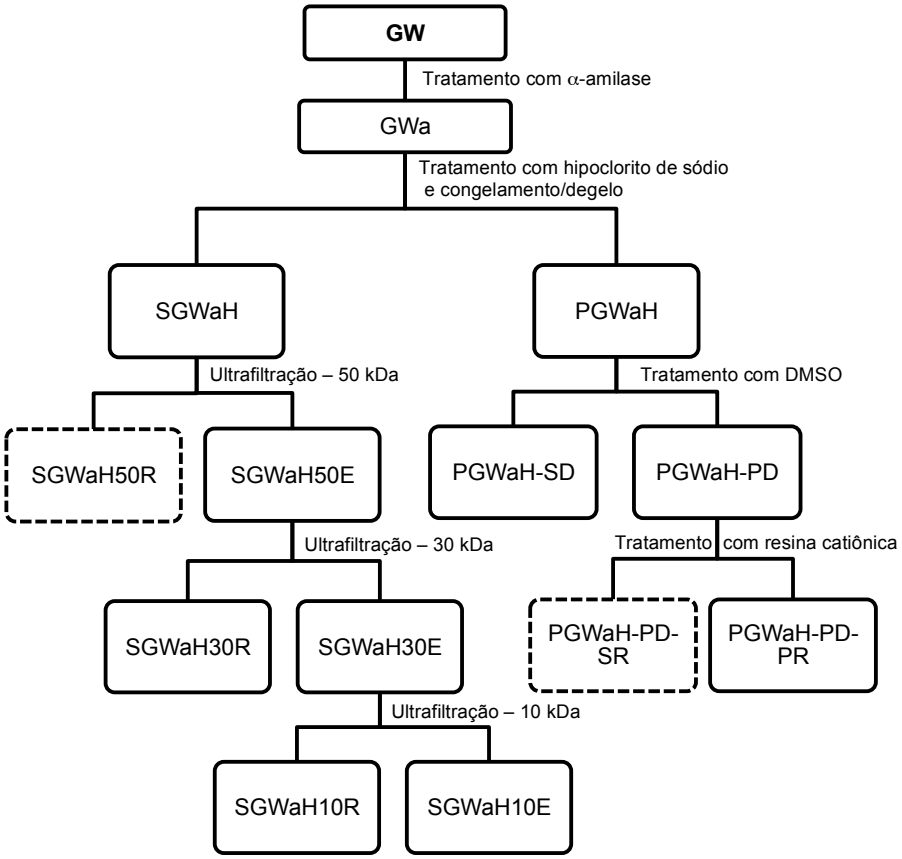

FIGURA 10 – ETAPAS DE PURIFICAÇÃO DOS POLISSACARÍDEOS OBTIDOS DA POLPA DA GRAVIOLA (*Annona muricata* L.) POR EXTRAÇÃO AQUOSA
FONTE: O autor (2015)

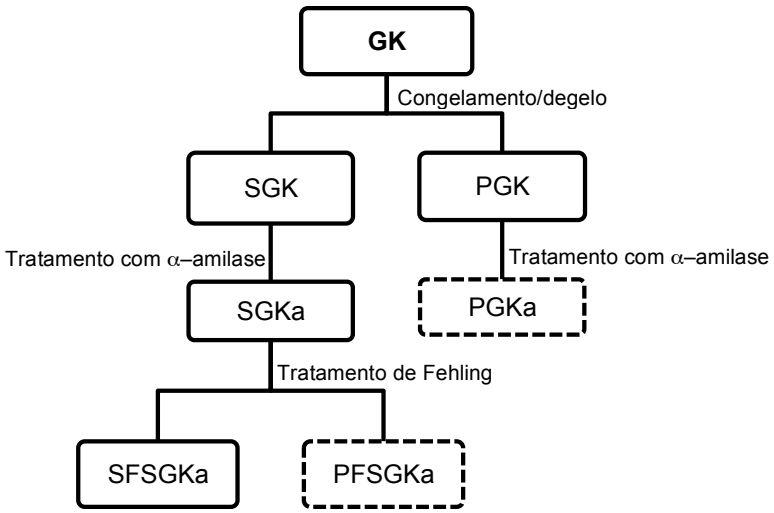

FIGURA 11 – ETAPAS DE PURIFICAÇÃO DOS POLISSACARÍDEOS OBTIDOS DA GRAVIOLA (*Annona muricata* L.) POR EXTRAÇÃO ALCALINA
FONTE: O autor (2015)

5.4.2 Tratamento com hipoclorito de sódio

A fração GWa (1 g) foi adicionada de 100 mL de NaOH (hidróxido de sódio) 0,1 M e 10 mL de NaClO (hipoclorito de sódio) e mantida sob agitação a temperatura ambiente por 12 horas. Posteriomente a fração foi neutralizada com ácido acético, dialisada (6-8 kDa / 24 horas) e submetida ao fracionamento por congelamente e degelo (FIGURA 10).

5.4.3 Fracionamento dos polissacarídeos por congelamento e degelo

As frações GWa (FIGURA 10) e GK (FIGURA 11) foram submetidas ao processo de congelamento e degelo. Nesse processo as amostras são solubilizadas em água destilada e posteriormente congeladas. Após o congelamento, as amostras são descongeladas em temperatura ambiente. As frações insolúveis em água fria foram separadas das frações solúveis por centrifugação (10.000 rpm / 20 min / 10 °C) (GORIN e IACOMINI, 1984), gerando frações sobrenadantes (SGWaH e SGK) e precipitadas (PGWaH e PGK). Esse processo foi repetido até que no sobrenadante não se formasse mais precipitado após o congelamento e degelo. Após esse processo, as frações foram concentradas e liofilizadas.

5.4.4 Tratamento com dimetilsulfóxido (DMSO)

A fração PGWaH (800 mg) foi adicionada de 15 mL de DMSO e mantida sob agitação a 50 °C por 12 horas, sendo posteriormente centrifugada (10.000 rpm / 15 min / 25 °C), dialisada (6-8 kDa / 24 horas) e liofilizada (FIGURA 10).

5.4.5 Fracionamento dos polissacarídeos pelo método de Fehling

A fração SGKa (FIGURA 11) foi submetida ao fracionamento pelo método de Fehling (JONES e STOODLEY, 1965). Inicialmente, a fração foi solubilizada em

água destilada e em seguida foi adicionado igual volume de uma mistura das soluções (1:1) de Fehling A e B, sendo a solução A composta por tartarato de sódio e potássio e KOH (173,0 g + 125 g / H_2O q.s.p. 500 mL) e a solução B composta de sulfato de cobre $CuSO_4.5H_2O$ (55,7 g / H_2O q.s.p. 500 mL). Após intensa agitação, foi mantida em refrigeração por 12 horas. O precipitado formado foi separado do sobrenadante por centrifugação (10.000 rpm / 20 min / 10 °C). O precipitado e o sobrenadante foram neutralizados com ácido acético glacial, dialisados em água corrente (12-14 kDa / 48 horas) e deionizados por resina catiônica, sendo novamente dialisados (12-14 kDa / 24 horas) e liofilizados.

5.4.6 Fracionamento dos polissacarídeos por ultrafiltração

A fração SGWaH foi submetida à ultrafiltração em membranas com limite de exclusão de 50, 30 e 10 kDa (Millipore®). As ultrafiltrações foram realizadas em um sistema de ultrafiltração (Sartorius®) acoplado a um cilindro de ar comprimido. A fração foi solubilizada em água destilada e após a ultrafiltração eram geradas uma fração eluída e uma retida na membrana (FIGURA 10).

5.5 CARACTERÍZAÇÃO ESTRUTURAL DOS POLISSACARÍDEOS

5.5.1 Composição Monossacarídica

A composição dos monossacarídeos neutros foi determinada após hidrólise ácida total de uma alíquota (2 mg) dos polissacarídeos com TFA (ácido trifluoracético) 1 M a 100 °C em estufa, por 16 horas. Após hidrólise, o ácido foi eliminado e o material resultante foi solubilizado em 1 mL de H_2O e submetido a conversão para alditóis a partir da redução com $NaBH_4$ (boroidreto de sódio) em pH alcalino, por no mínimo 12 horas a temperatura ambiente (WOLFROM e THOMPSOM, 1963a). Após, o material foi neutralizado com ácido acético e liofilizado. Os derivados alditóis obtidos foram acetilados com uma mistura de anidrido acético-piridina (1:1, v/v; 1 mL), à temperatura ambiente, *overnight*,

formando os acetatos de alditóis. O processo foi interrompido com a adição de água e os acetatos de alditóis extraídos com clorofórmio. A piridina residual presente na fração clorofórmica foi removida com sucessivas lavagens com solução de $CuSO_4$ (sulfato de cobre) a 5%. Após remoção total da piridina, a fração clorofórmica foi desidratada com sulfato de sódio anidro, filtrada e evaporada até secura em temperatura ambiente (WOLFROM e THOMPSOM, 1963b). Os acetatos de alditóis produzidos foram analisados por GC-MS e identificados pelo seus tempos de retenção e perfis de fragmentação.

A determinação de ácidos urônicos foi realizada conforme descrito por Filisetti-Cozzi e Carpita (1991) usando meta-hidroxibifenila (0,15% p/v em NaOH 0,5% p/v). A leitura foi realizada em espectrofotômetro a 525 nm. O ácido galacturônico foi utilizado como padrão.

5.5.2 Determinação da homogeneidade e massa molar

As análises de homogeneidade e a determinação da massa molar foram realizadas em HPSEC-MALLS. As amostras foram solubilizadas em $NaNO_2$ 0,1 M para uma concentração final de 1 mg/mL e filtradas em membranas de éster de celulose (Millipore®), de porosidade 0,22 µm.

Para o cálculo da massa molar, padrões de dextrana (487 kDa, 266 kDa, 124 kDa, 72,2 kDa, 40,2 kDa, 17,2 kDa e 9,4 kDa - SIGMA®) foram utilizadas na obtenção de uma curva de calibração. A massa molar das amostras foi calculada de acordo com a curva de calibração.

5.5.3 Ressonância magnética nuclear (RMN)

Os espectros de RMN de ^{13}C foram obtidos em espectrômetro Bruker Avance III 400 MHz, do Centro de RMN da Universidade Federal do Paraná. Os deslocamentos químicos foram expressos em ppm, e foram utilizados como padrão interno os sinais de acetona (δ 30,2) ou DMSO-d_6 (δ 39,7). As análises foram

realizadas a 50 °C e as amostras foram solubilizadas em D_2O ou Me_2SO-d_6, de acordo com a solubilidade.

5.6 RESULTADOS E DISCUSSÃO DOS POLISSACARÍDEOS DA POLPA GRAVIOLA

A amostra deslipidificada (498,6 g) da graviola foi submetida a extrações aquosas sequenciais, gerando a fração GW com 31% de rendimento. A composição monossacarídica da fração GW apresentou uma alta concentração de glucose (73%) (TABELA 1) e uma coloração azul no teste de Lugol, sugerindo a presença de amido na fração. O espectro de RMN-^{13}C da fração GW está demonstrado na Figura 12 e apresentou os sinais em δ 100,2 (C-1), δ 72,1 (C-2), δ 73,3 (C-3), δ 79,0 (C-4 substituído), δ 71,7 (C-5) e δ 60,7 (C-6) atribuídos às unidades de α-D-Glc*p* (1→4)-ligadas (VRIESMANN; SILVEIRA; PETKOWICZ, 2009), confirmando a presença de amido na fração. Portanto, a fração GW foi tratada com α-amilase de *Bacillus licheniformis* (SIGMA®) para remoção do amido (FIGURA 10), gerando a fração GWa. Essa fração foi tratada com solução de hipoclorito de sódio e submetida ao processo de congelamento e degelo, gerando as frações SGWaH (solúvel em água fria) e PGWaH (insolúvel em água fria) (FIGURA 10).

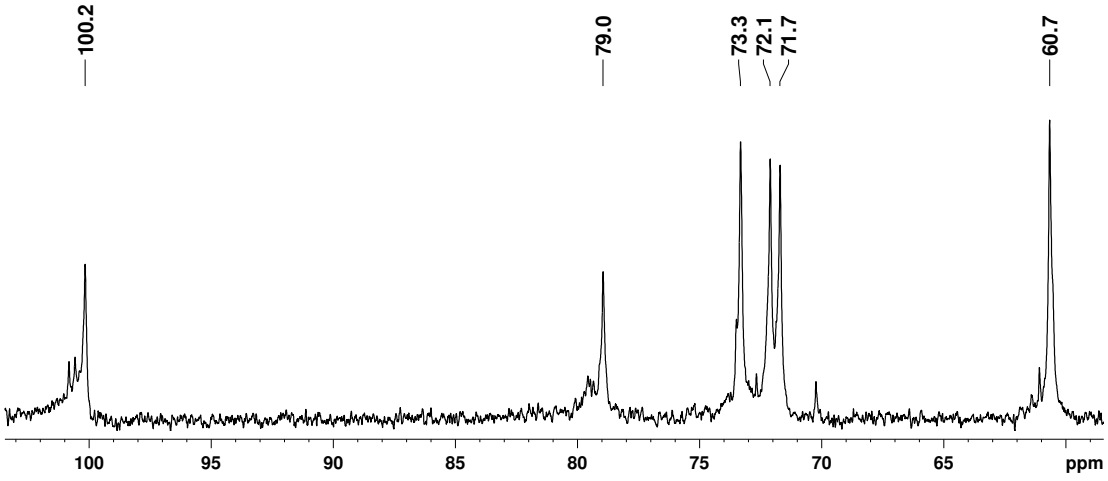

FIGURA 12 – ESPECTRO DE RMN-^{13}C DA FRAÇÃO GW. EXPERIMENTO REALIZADO EM DMSO-d_6 A 50 °C, COM DESLOCAMENTOS QUÍMICOS (δ) EXPRESSOS EM PPM
FONTE: O autor (2015)

A fração SGWaH apresentou perfil de eluição em HPSEC heterogêneo e o espectro de RMN-^{13}C (dados não mostrados) mostrou-se bastante complexo, portanto, a fração foi submetida à ultrafiltração em membranas (Millipore®) com limite de exclusão de 50 kDa, 30 kDa e 10 kDa (FIGURA 10). A fração SGWaH50R apresentou perfil homogêneo em HPSEC (FIGURA 13) e massa molar de 74 kDa. A composição monossacarídica desta fração mostrou principalmente galactose e arabinose (TABELA 1).

TABELA 1 – COMPOSIÇÃO MONOSSACARIDICA DAS FRAÇÕES DERIVADAS DA EXTRAÇÃO AQUOSA DA POLPA DA GRAVIOLA

FRAÇÕES	RENDIMENTOS[1]		MONOSSACARIDEOS (%)						
	g	%	Rha	Fuc	Ara	Xyl	Gal	Glc	AU[2]
GW	192,0	31,0	-	-	11,6	6,2	-	72,7	10,5
GWa	53,0	8,5	2,3	-	23,7	2,4	3,5	35,5	32,5
SGWaH50R	1,2	0,2	3,3	2,6	29,4	2,6	46,2	4,1	11,8
PGWaH-PD-SR	4,0	0,7	2,1	-	5,9	-	2,1	9,5	75,35

NOTAS: Análise de acetatos de alditóis por GC-MS. (1) Partindo de 619 g de polpa seca; (2) Determinação de ácidos urônicos segundo Filisetti-Cozzi e Carpita (1991).

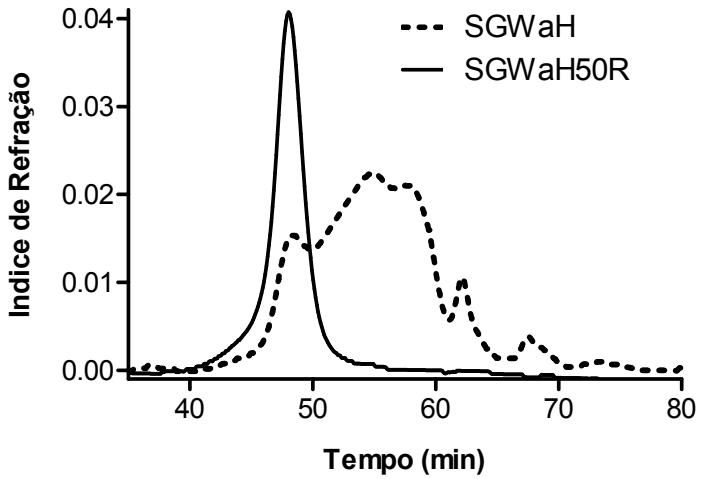

FIGURA 13 – PERFIS DE ELUIÇÃO EM HPSEC DAS FRAÇÕES SGWAH E SGWAH50R, UTILIZANDO DETECTOR DE ÍNDICE DE REFRAÇÃO (IR)
FONTE: O autor (2015)

O espectro de RMN-^{13}C da fração SGWaH50R (FIGURA 14) apresentou diversos sinais na região anomérica (δ 98,4 – 109,1). Os sinais em δ 106,4, 107,0, 107,5, 107,9 e 109,1 correspondem as unidades de α-L-Ara*f*. O sinal em δ 103,4 corresponde aos C-1 de unidades de β-D-Gal*p* (1→3) e (1→3,6)-ligadas. O sinal em δ 103,1 é atribuído às unidades de β-D-Gal*p* (1→6)-ligadas (CIPRIANI *et al*., 2006; BENTO; NOLETO; PETKOWICZ, 2014). Os sinais em δ 69,2, 61,3 e 61,0 provavelmente pertecem aos C-6 substituído e não substituído das unidades de β-D-Gal*p* e ao C-5 das unidades de α-L-Ara*f* não substituído, respectivamente (DELGOBO *et al*., 1999). Estes assinalamentos sugerem a presença de arabinogalactana do tipo II na fração. Simas-Tosin *et al.* (2012) também verificaram a presença de AG-II em polpa de pêssego. Além destes sinais, podemos observar sinais em δ 102,6, 99,3 e 98,4 que podem ser atribuídos aos carbonos anoméricos das unidades de β-D-Glc*p*, α-L-Fuc*p* e α-D-Xyl*p*. O sinal em δ 15,4 pode ser atribuídos ao C-6 das unidades de α-L-Fuc*p* (BUSATO *et al*., 2005). Esses dados estão de acordo com a composição monossacarídica (TABELA 1) onde podemos observar menores porcentagens de glucose, fucose e xilose, sugerindo a presença de traços de xiloglucana nesta fração. As frações SGWaH30R, SGWaH10R E SGWaH10E não foram analisadas devido ao baixo rendimento.

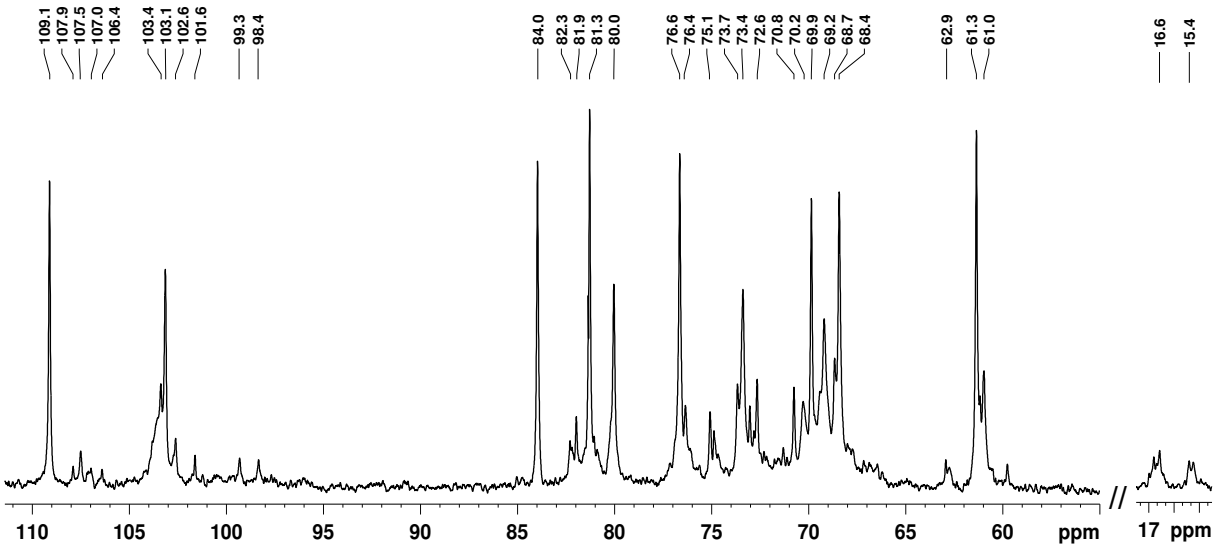

FIGURA 14 – ESPECTRO DE RMN-^{13}C DA FRAÇÃO SGWAH50R. EXPERIMENTO REALIZADO EM D_2O A 50 °C, COM DESLOCAMENTOS QUÍMICOS (δ) EXPRESSOS EM PPM
FONTE: O autor (2015)

A fração PGWaH (insolúvel em água fria) foi submetida a um tratamento com DMSO, gerando as frações PGWaH-SD (sobrenadante do tratamento com DMSO) e PGWaH-PD (precipitado do tratamento com DMSO) (FIGURA 10). A fração PGWaH-SD foi analisada por RMN-^{13}C (FIGURA 15) e apresentou sinais em δ 100,2 (C-1), δ 72,2 (C-2), δ 73,4 (C-3), δ 79,0 (C-4 substituído), δ 71,8 (C-5) e δ 60,7 (C-6) atribuídos às unidades de α-D-Glc*p* (1→4)-ligadas (VRIESMANN; SILVEIRA; PETKOWICZ, 2009), indicando a presença de amido na fração.

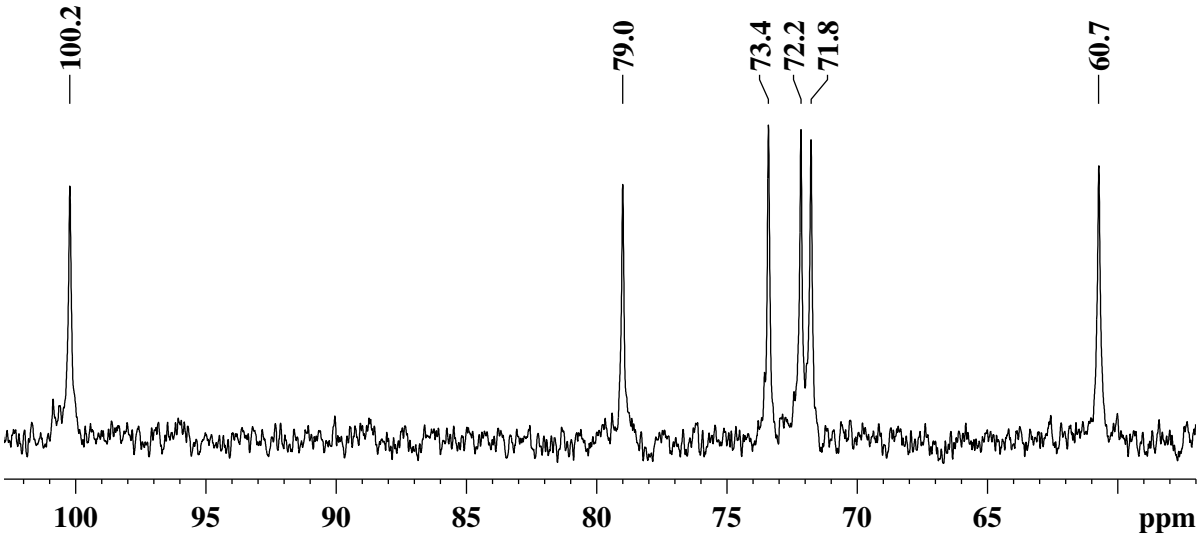

FIGURA 15 – ESPECTRO DE RMN-^{13}C DA FRAÇÃO PGWaH-SD. EXPERIMENTO REALIZADO EM DMSO-d_6 A 50 °C, COM DESLOCAMENTOS QUÍMICOS (δ) EXPRESSOS EM PPM
FONTE: O autor (2015)

A fração PGWaH-PD apresentou-se insolúvel em água e DMSO e por isso foi dispersa em água e tratada com resina catiônica, gerando as frações PGWaH-PD-SR (sobrenadante do tratamento com resina catiônica) e PGWaH-PD-PR (precipitado do tratamento com resina catiônica) (FIGURA 10).

A fração PGWaH-PD-SR apresentou perfil homogêneo em HPSEC (FIGURA 16) com massa molar de 16 kDa. O espectro de RMN-^{13}C da fração PGWaH-PD-SR está demonstrado na Figura 17 e apresentou os sinais em δ 98,9 (C-1), δ 68,9 (C-2), δ 68,3 (C-3), δ 77,9 (C-4 substituído), δ 71,2 (C-5) e δ 174,9 (COOH) atribuídos a unidades de α-D-Gal*p*A (1→4)-ligadas (CANTU-JUNGLES *et al*., 2014), indicando a presença de uma homogalacturonana linear na fração. A presença de

homogalacturonana linear foi reportada em polpa de pêssego (SIMAS-TOSIN *et al.*, 2012).

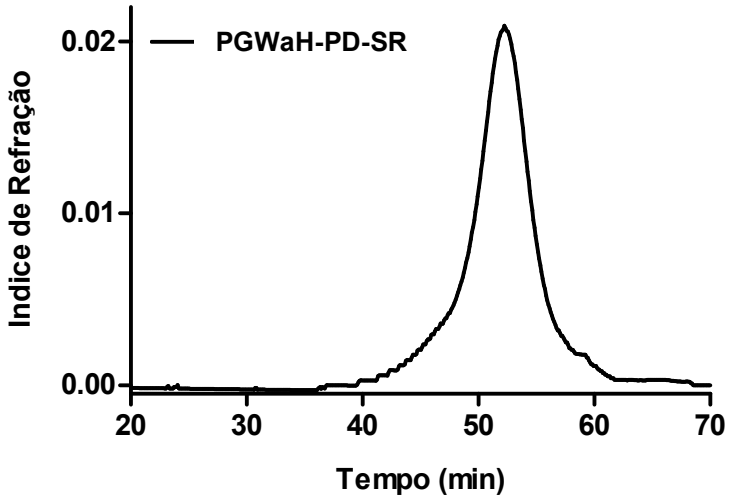

FIGURA 16 – PERFIL DE ELUIÇÃO EM HPSEC DA FRAÇÃO PGWAH-PD-SR, UTILIZANDO DETECTOR DE ÍNDICE DE REFRAÇÃO (IR)
FONTE: O autor (2015)

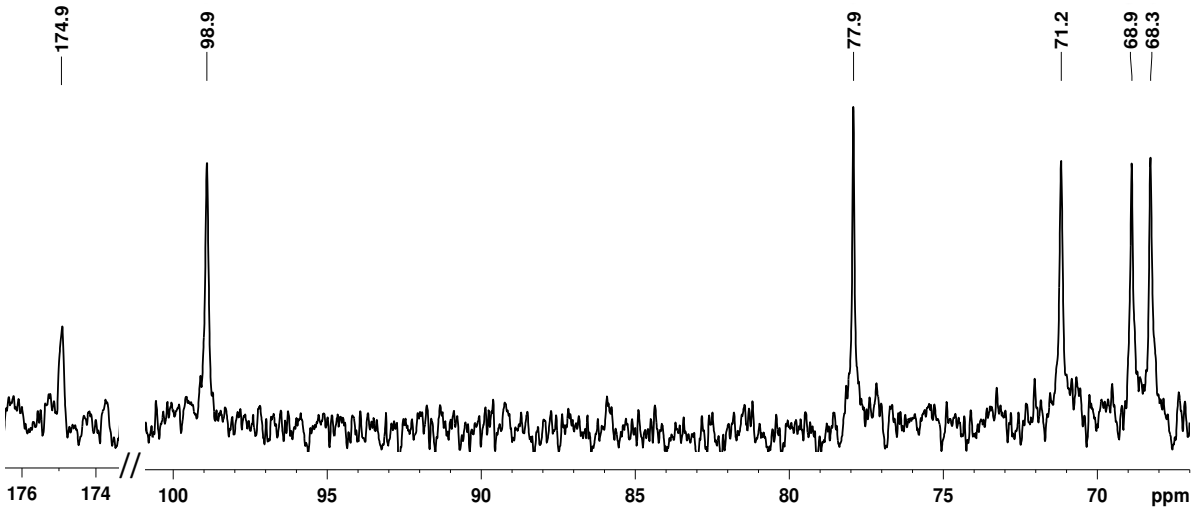

FIGURA 17– ESPECTRO DE RMN-^{13}C DA FRAÇÃO PGWAH-PD-SR. EXPERIMENTO REALIZADO EM DMSO-d_6 A 50 °C, COM DESLOCAMENTOS QUÍMICOS (δ) EXPRESSOS EM PPM
FONTE: O autor (2015)

O resíduo da extração aquosa foi submetido à extrações alcalinas com NaOH 10%, gerando a fração GK (FIGURA 11). A fração GK foi submetida ao processo de congelamento e degelo, resultando nas frações SGK (solúvel em água fria) e PGK (insolúvel em água fria) (FIGURA 11). As frações SGK e PGK apresentaram alta concentração de glucose na composição monossacarídica (TABELA 2), sugerindo a presença de amido nas frações, a qual foi confirmada pelos espectros de RMN-^{13}C (dados não mostrados) destas frações. Portanto, elas foram tratadas com α-amilase de *Bacillus licheniformis* (SIGMA®) para remoção do amido, gerando as frações SGKa e PGKa (FIGURA 11).

A fração PGKa, após tratamento com α-amilase (2 vezes), apresentou-se composta apenas por xilose (TABELA 2) e o espectro de RMN-^{13}C dessa fração (FIGURA 18) apresentou os sinais em δ 101,9 (C-1), δ 72,7 (C-2), δ 74,1 (C-3), δ 75,6 (C-4) e δ 63,3 (C-5) atribuídos a unidades de β-D-Xyl*p* (1→4)-ligadas (CORDEIRO; ALMEIDA; IACOMINI, 2015; HABIBI; MAHROUZ; VIGNON, 2002). Esses assinalamentos sugerem a presença de uma β-D-(1→4)-xilana linear nessa fração. A presença de β-D-(1→4)-xilana linear em frutas foi reportada por Cordeiro, Almeida e Iacomini (2015) na polpa do buriti.

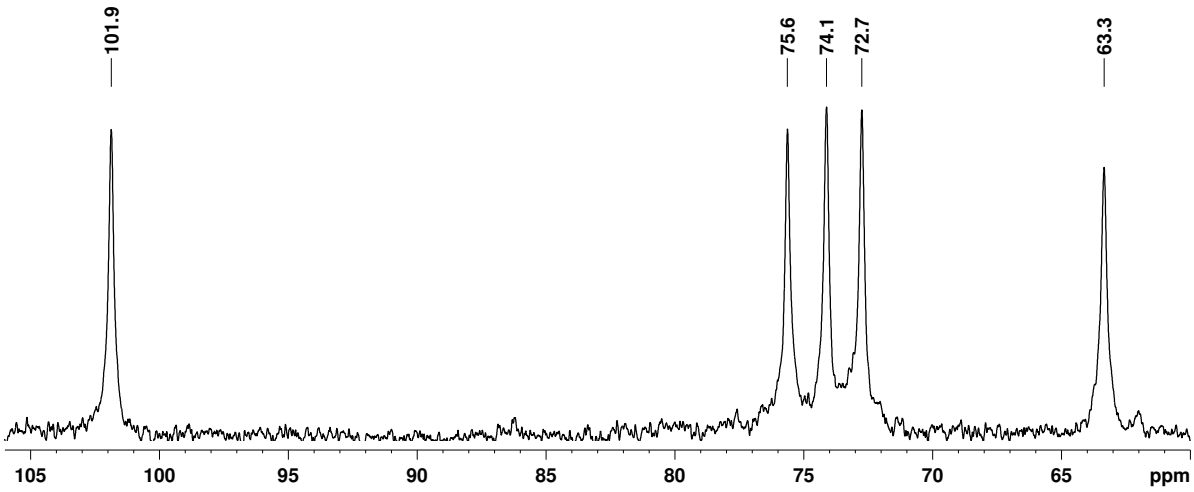

FIGURA 18 – ESPECTRO DE RMN-^{13}C DA FRAÇÃO PGKa. EXPERIMENTO REALIZADO EM DMSO-d_6 A 50 °C, COM DESLOCAMENTOS QUÍMICOS (δ) EXPRESSOS EM PPM
FONTE: O autor (2015)

A fração SGKa apresentou na composição monossacarídica 22,8% de glucose (TABELA 2), a qual pode estar relacionada à presença de amido, mesmo após o tratamento com α-amilase. No entanto, não foi realizado um outro tratamento enzimático. A fração SGKa foi submetida ao tratamento com solução de Fehling, gerando as frações SFSGKa (sobrenadante do tratamento de Fehling) e PFSGKa (precipitado do tratamento de Fehling) (FIGURA 11). A fração SFSGKa apresentou arabinose como principal monossacarídeo, além de menores porcentagens de galactose, glucose, ramnose e ácidos urônicos (TABELA 2). A fração PFSGKa apresentou na análise de composição monossacarídica, principalmente glucose e menores porcentagens de xilose, galactose, arabinose, fucose e ácidos urônicos (TABELA 2).

TABELA 2 – COMPOSIÇÃO MONOSSACARÍDICA DAS FRAÇÕES DERIVADAS DA EXTRAÇÃO ALCALINA DA POLPA DA GRAVIOLA

FRAÇÕES	RENDIMENTOS[1]		MONOSSACARIDEOS (%)							
	g	%	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	AU[2]
GK	35,0	5,6	4,4	0,6	32,0	18,9	1,2	4,5	38,4	nd [3]
PGK	13,0	2,1	-	-	3,6	7,3	-	-	87,7	1,4
PGKa	4,3	0,7	-	-	-	100	-	-	-	-
SGK	13,4	2,2	4,1	3,3	27,0	13,6	5,2	8,0	38,8	nd
SGKa	10,1	1,6	2,8	4,9	33,5	14,9	3,4	12,4	22,8	5,4
SFSGKa	3,2	0,5	5,1	-	63,0	3,4	-	12,1	8,0	8,4
PFSGKa	5,4	0,9	-	6,0	8,3	22,7	-	14,2	44,1	4,7

NOTAS: Análise de acetatos de alditóis por GC-MS. (1) Partindo de 619 g de polpa seca; (2) Determinação de ácidos urônicos segundo Filisetti-Cozzi e Carpita (1991); (3) Não determinado.

A fração PFSGKa apresentou perfil heterogêneo em HPSEC (FIGURA 19) e o espectro de RMN-^{13}C dessa fração (FIGURA 20) contém uma diversidade de sinais na região anomérica. Os sinais em δ 104,4 e 103,2 podem ser atribuídos ao C-1 de unidades de β-D-Gal*p*, enquanto que o sinal em δ 102,3 pode ser atribuído ao C-1 de unidades de β-D-Glc*p*. Os sinais anoméricos em δ 99,4 e 98,9 podem ser

atribuídos às unidades de α-L-Fuc*p* e α-D-Xyl*p,* respectivamente. O sinal em δ 15,9 pode ser atribuído ao C-6 das unidades de α-L-Fuc*p*. Estes assinalamentos estão de acordo com a literatura (BUSATO *et al.*, 2005) e sugerem a presença de uma xiloglucana na fração. Além destes sinais, podemos observar o sinal em δ 101,6 que pode ser atribuído ao C-1 de unidades de β-D-Xyl*p* (CORDEIRO; ALMEIDA; IACOMINI, 2015). Este sinal pode indicar a presença de traços de β-xilana na fração. Por outro lado, xiloglucanas com cadeias laterais contendo resíduos de β-D-Xyl*p* já foram descritas na literatura (FIGURA 9) para folhas de argania (*Argania spinosa*) (RAY *et al*., 2004) e em frutos, como o mirtilo (HILZ *et al*., 2007).

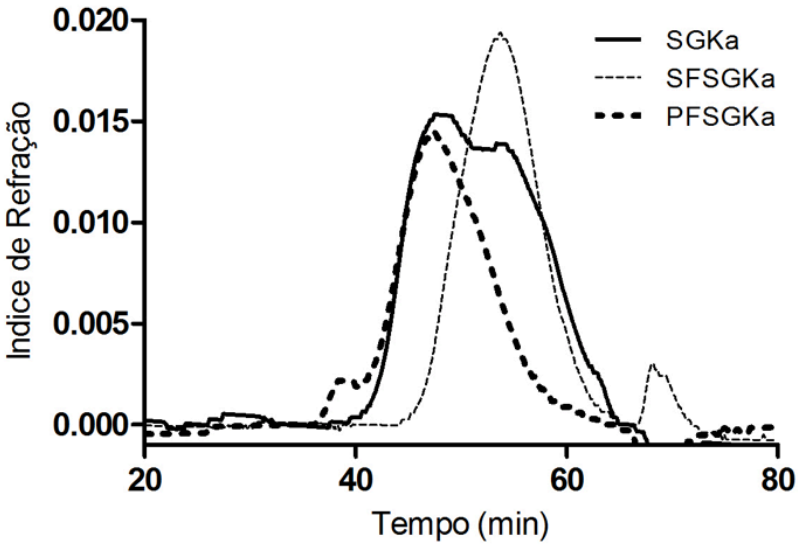

FIGURA 19 – PERFIS DE ELUIÇÃO EM HPSEC DA FRAÇÕES SGKa, SFSGKa E PFSGKa, UTILIZANDO DETECTOR DE ÍNDICE DE REFRAÇÃO (IR)
FONTE: O autor (2015)

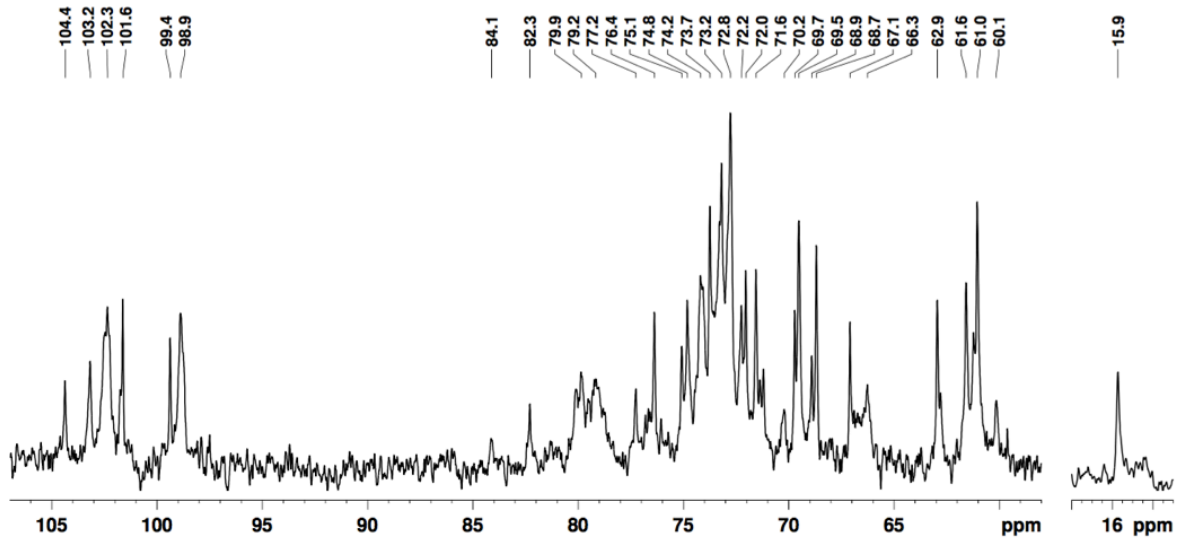

FIGURA 20 – ESPECTRO DE RMN-^{13}C DA FRAÇÃO PFSGKa. EXPERIMENTO REALIZADO EM D_2O A 50 °C, COM DESLOCAMENTOS QUÍMICOS (δ) EXPRESSOS EM PPM
FONTE: O autor (2015)

CONCLUSÕES

Polissacarídeos pécticos e hemicelulósicos dos frutos da carambola foram obtidos a partir de extrações aquosa e alcalina. Eles foram caracterizados por análises de composição monossacarídica, RMN, HPSEC e metilação como sendo:

- Ramnogalacturonana tipo I com inserções de arabinogalactana tipo I e arabinana proveniente do extrato alcalino;
- Fucogalactoxiloglucana e heteroxilana proveniente do extrato alcalino;
- Duas frações pécticas contendo arabinogalactana tipo II proveniente do extrato aquoso;
- Galacturonana substituída proveniente do extrato aquoso;

A galacturonana substituída apresentou propriedades antinociceptivas e anti-inflamatórias em modelo de edema de pata induzido por formalina, apresentando 99% de redução da dor na segunda fase do teste na dose de 300 mg/kg e também reduziu o edema de pata em 53%, sugerindo que o efeito antinociceptivo foi decorrente do efeito anti-inflamátorio.

Em frações obtidas a partir de extrações aquosa e alcalina da polpa dos frutos da graviola, foram encontradas evidências da presença dos seguintes polissacarídeos:

- Amido proveniente dos extratos aquoso e alcalino;
- Homogalacturonana linear e arabinogalactana tipo II proveniente do extrato aquoso;
- Xilana linear β-(1→4)-ligada e xiloglucana proveniente de extrato alcalino;

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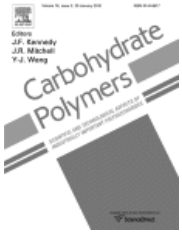
ANEXOS



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